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Incorporation of Radioactive Phosphate into the Nucleic Acids of Brain Slices

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(*Received* 31 *January* 1953)

The unequivocal incorporation of radioactive phosphate (^{32}P) into the nucleic acids of brain slices has not as yet been demonstrated. Because of the great interest in the nucleic acids of the nervous system aroused by the work of Caspersson (1947) and Hydén (1943, 1947), it was decided to investigate the possible incorporation of ^{32}P into the pentosenucleic acid (PNA) and deoxypentosenucleic acid (DNA) of slices of brain tissue respiring in a suitable oxygenated medium.

In experiments with tissue slices only a small amount of material is available. For this reason most previous workers carrying out *in vitro* experiments have resorted to the method of Schmidt & Thannhauser (1945) for the separation of PNA and DNA. It has been shown by Jeener (1949), Davidson, Frazer & Hutchinson (1951), Szafarz & Paternotte (1951), and others, in experiments in which animals were killed after the administration of ^{32}P, that the specific activity of the Schmidt-Thannhauser PNA fraction of liver is much greater than that of more purified PNA preparations from the same tissue, even after the very active orthophosphate fraction, presumably derived from 'phosphoprotein', has been removed. Strickland (1952) reported similar findings for brain tissue. With tissue from the nervous system the situation is even less favourable than with liver, for Logan, Mannell & Rossiter (1952) have shown that more than one-half of the protein-bound phosphorus of brain tissue appears in the Schmidt-Thannhauser hydrolysate in the form of organic phosphate that has not the characteristic ultraviolet absorption of nucleotides. In white matter of brain this non-nucleotide phosphorus, which has a specific activity much greater than that of PNA, may account for as much as 80% of the phosphorus in the Schmidt-Thannhauser PNA fraction. Much of this phosphorus may be derived from the inositol-containing complex isolated from brain by Folch & Le Baron (1951), but other active phosphorus compounds may be present also. Davidson & Smellie (1952*b*) have recently shown that the Schmidt-Thannhauser PNA fraction of rat-liver tissue contains at least six non-nucleotide phosphate derivatives, which together make up 25% of the phosphorus of the fraction. Two hours after an injection of ^{32}P the specific activity of each of these fractions was greater than that of the isolated PNA nucleotides.

In view of these difficulties encountered when ^{32}P was administered *in vivo*, it is not surprising that similar difficulties arose when ^{32}P was added to brain slices respiring *in vitro*. In experiments with tissue slices it is convenient to add sufficient ^{32}P to make the specific activity of the inorganic phosphate of the incubating medium greater than the specific activity usually observed for the inorganic phosphate fraction of tissues from animals that have received ^{32}P *in vivo*. This presumably leads to a greater specific activity of the highly active contaminants present in the Schmidt-Thannhauser PNA fraction.

In the experiments described in this paper the nucleic acids were removed from the slices by the method of Hammarsten (1947), which separates them from most of the other active constituents (Strickland, 1952). The nucleotides of the PNA were then removed from the DNA by the alkaline hydrolysis procedure of Schmidt & Thannhauser (1945). The nucleotides so obtained were separated by paper chromatography, using the techniques of Markham & Smith (1949, 1951) and Magasanik, Vischer, Doniger, Elson & Chargaff (1950). For specific activity measurements the individual nucleotides were eluted from the chromatograms. Similar chromatograms were photographed with ultraviolet light, after which they were exposed to an X-ray film for radioautography.

A preliminary account of this work has appeared (Strickland, DeLuca & Rossiter, 1953).

METHODS

Preparation and incubation of brain slices. Slices were cut with a safety-razor blade from the cerebral hemispheres of a cat killed by decapitation. Each slice was rapidly weighed on a torsion balance and transferred to a standard Warburg reaction vessel containing 5 ml. Krebs-Ringer bicarbonate incubating medium, to which glucose had been added to make a final concentration of 0·2% (w/v). Sufficient slices were cut to give a weight of 500 mg. in each vessel. To each sample was added 24 μc. ^{32}P supplied in the form of $H_2{}^{32}PO_4$ by the Eldorado Mining and Refining (1944) Ltd. The vessels were filled with a 95% O_2 and 5% CO_2 mixture, after which they were incubated in a bath at 37·2° for 4 hr. During the

* Ontario Research Council Scholar.

incubation period the vessels were shaken in the usual way to ensure proper oxygenation of the incubating medium.

The contents of each flask were dispersed at 0° in 10 ml. 10% (w/v) trichloroacetic acid (TCA) with a Potter-Elvehjem type homogenizer. The suspension was centrifuged and the supernatant was discarded. To remove last traces of contaminating acid-soluble phosphorus, the residue was extracted seven times with carrier phosphate (5 ml. 0·04 M-KH_2PO_4 in 10% TCA) at 0–4° and finally twice with 5 ml. 10% TCA. The final extracts contained negligible activity. The lipid phosphorus was removed by two cold extractions with 5 ml. 95% (v/v) ethanol, four extractions with 5 ml. ethanol:ether, (3:1, v/v) heated to boiling, and the residue was finally washed with 5 ml. ether. All experiments were done in duplicate. For experiments at 0 hr. the ^{32}P was added to the flask containing the brain slices. The flask was shaken to ensure thorough mixing and the entire contents, including the ^{32}P, were then dispersed at 0° in 10 ml. 10% TCA as described for the incubated slices.

Extraction of nucleic acids by method of Hammarsten (1947)

The nucleic acids were extracted from the protein residue as follows: 1·0 ml. of 10 M-urea was pipetted on to the residue and allowed to stand for 5 min. To this was added 3·0 ml. of S solution (a saturated solution of NaCl saturated with $(NH_4)_2SO_4$ at a boiling temperature and allowed to cool). The nucleic acids were then extracted by heating the solution to a boiling temperature for 1 min. Three more extractions were carried out with 2·0 ml. of S-urea solution (150 g. urea/l. S solution) at boiling temperature for 1 min. The nucleic acids were precipitated from the combined extracts by the addition of 1·0 ml. saturated $CuSO_4$. After standing overnight the tube was centrifuged and the supernatant discarded. The precipitate was washed twice with 1·8% $CuCl_2$ and then the copper nucleates were decomposed and the nucleic acids were extracted by successive additions (0·8 ml. and three 0·4 ml. portions) of a freshly prepared 1:1 mixture of acetate buffer, pH 6·4 (100 g. potassium acetate/100 ml. water titrated to pH 6·4 with glacial acetic acid) and 10 M-urea. This was followed by three extractions using 0·2 ml. 5 M-urea. The nucleic acids were then precipitated at −15° by the addition of 10 ml. 95% (v/v) ethanol and 1 drop saturated NaCl to the combined extracts. The precipitate was redissolved in 1·0 ml. water and re-precipitated by the addition of 0·11 ml. N-HCl at 0° followed by 10 ml. acid-alcohol (95% ethanol: N-HCl, 9:1, v/v) at −15° for at least 2 hr. This precipitate was dissolved in 0·01 N-NaOH and the nucleic acids were precipitated as before. This precipitate was then separated into DNA and PNA by the method of Schmidt & Thannhauser (1945). The precipitated DNA was washed with 5% TCA and the nucleotides of the PNA subjected to the magnesia treatment described by Friedkin & Lehninger (1949). Both fractions were then ashed and the specific activities determined as described below. The recovery of the total amount of nucleic acid in the tissue was of the order of 70–80%.

Chromatography

For the chromatographic experiments the precipitates of mixed DNA and PNA from five reaction vessels were pooled and hydrolysed overnight in 0·15 ml. N-NaOH at room temperature to liberate the PNA nucleotides. The hydrolysate was brought to pH 8 with HCl. The DNA was not precipitated, since it did not interfere with the separation of the nucleotides. Portions of the hydrolysate (10 μl.) were applied to filter-paper strips (19 × 45 cm. no. 597, Schleicher and Schüll). The assembly was as described by Magasanik *et al.* (1950). Either two or three chromatograms were run in each of the following solvents:

(1) *iso*Butyric acid, 10 vol., and 0·5 N-ammonia solution, 6 vol. Two 30 ml. portions were placed in flat dishes at the bottom of the tank. The solvent system was that described by Magasanik *et al.* (1950). It separated adenylic acid and cytidylic acid from the other nucleotides. The running time was 16–18 hr.

(2) *tert.*-Butanol, 70%, water, 30% (v/v) and enough HCl to make the solvent 0·8 M. Two 30 ml. portions of the solvent were placed in flat dishes at the bottom of the tank. This solvent, described by Smith & Markham (1950), was used to separate uridylic acid from the other nucleotides. The running time was about 24 hr.

(3) Saturated $(NH_4)_2SO_4$, 79%, 0·2 M-acetate buffer, pH 5·9, 19%, and *iso*propanol, 2% (v/v/v). Two 30 ml. portions of the solvent with excess *iso*propanol were placed in flat dishes at the bottom of the tank. This solvent system, described by Markham & Smith (1951), was used to separate the isomeric adenylic acids *a* and *b* and guanylic acids *a* and *b* from the other nucleotides. The running time was 6 to 8 hr.

Ultraviolet photography and radioautography

The nucleotides were localized on the chromatograms with a 'Mineralight' short-wave ultraviolet lamp. Photographs of the chromatograms were made with an assembly similar to that described by Markham & Smith (1949, 1951). An 8 W germicidal lamp (General Electric) emitting a high percentage of ultraviolet light at 254 mμ. was used as a light source. The light beam was filtered through a Corning no. 9863 filter and a cobalt-nickel liquid filter ($CoSO_4$, $7H_2O$, 7 g. and $NiSO_4$. $7H_2O$, 23 g.) contained in a round-bottomed quartz flask. The chromatogram was placed in contact with Kodagraph Contact paper and exposed for 3 min. at 6 ft.

The chromatograms were then placed in contact with a strip of X-ray 'No Screen Film' for times varying from 17 to 28 days according to the activity of the nucleotides.

Elution of nucleotides

The nucleotide spots located with the 'Mineralight' lamp were marked out in pencil. The spots, with comparable spots from blank lanes, were cut out and eluted with 5 ml. 0·1 N-HCl for 18 hr. The extinction of the eluted nucleotides was measured at $\lambda_{max.}$ in the Beckman DU spectrophotometer. The following are the molecular extinction coefficients used and the $\lambda_{max.}$ found:

	$\lambda_{max.}$	ϵ
Adenylic acid	258	13 900
Guanylic acid	257	11 800
Cytidylic acid	278	12 700
Uridylic acid	261	9 900

The values for the extinction coefficients are those reported by Marrian, Spicer, Balis & Brown (1951) for the purine nucleotides and those reported by Ploeser & Loring (1949) for the pyrimidine nucleotides. They do not differ greatly from those reported by Holiday (1930) or from those used by Markham & Smith (1951). The values found for

$\lambda_{max.}$ of each of the nucleotides are in agreement with those reported in the literature.

From the concentrations of the nucleotide in the eluate the molar ratios of the nucleotides were calculated. The figures have a relative significance only, for the separation procedure of Hammarsten (1947) is not quantitative. This lack of quantitative recovery is offset by the finding that the method yields a PNA preparation sufficiently free from contaminating substances for satisfactory specific activity determinations.

Specific activity measurements

A portion of the solution of each of the eluted nucleotides, or of the solution of PNA or DNA separated by the method of Hammarsten (1947), was ashed with 60% perchloric acid. The inorganic phosphate and the radioactivity of the resulting inorganic phosphate was determined by the method of Ernster, Zetterström & Lindberg (1950). The counting was done with a liquid counter (20th Century Electronics, M-6).

RESULTS

Comparison of methods of Schmidt & Thannhauser (1945) and Hammarsten (1947)

In the experiments reported in Table 1 the PNA from similar slices of brain from the same animal was separated either by the standard procedure of Schmidt & Thannhauser (1945) or by the method of Hammarsten (1947). The Schmidt-Thannhauser hydrolysate was then subjected to the magnesia treatment described by Friedkin & Lehninger (1949) to remove inorganic phosphate. It can be seen that in each instance the activity of the fraction from the incubated slices was much greater than that of the corresponding fraction from the 0 hr. control. The magnesia treatment reduced the activity of both the unincubated and the incubated slices, but not to levels approaching the values given by the Hammarsten method. The reason for this is the high concentration of active non-nucleotide ester phosphorus in the Schmidt & Thannhauser hydrolysate (Logan *et al.* 1952; Strickland, 1952).

Table 1. *Specific activity of PNA of cat-brain slices incubated at 37·2° in Krebs-Ringer bicarbonate containing* ^{32}P. *Glucose*, 0·011 M.

(Counts/min./μg. P.)

	Expt. 1		Expt. 2	
	0 hr.	4 hr.	0 hr.	4 hr.
Schmidt-Thannhauser (1945) hydrolysate	2·7	281	5·7	354
Schmidt-Thannhauser (1945) hydrolysate after magnesia treatment	0·4	141	0·1	162
Hammarsten (1947)	0·3	17·9	0	22·5

Table 2 shows similar data for DNA. Again it can be seen that the magnesia treatment reduced the activity of the Schmidt-Thannhauser DNA, but it was not reduced to figures comparable to those found for the DNA separated by the Hammarsten method.

Table 2. *Specific activity of DNA of cat-brain slices incubated at 37·2° in Krebs-Ringer bicarbonate containing* ^{32}P. *Glucose*, 0·011 M.

(Counts/min./μg. P.)

	Expt. 1		Expt. 2	
	0 hr.	4 hr.	0 hr.	4 hr.
Schmidt-Thannhauser (1945)	0·3	5·3	0	12·7
Schmidt-Thannhauser (1945) after magnesia treatment	—	3·4	0·6	6·6
Hammarsten (1947)	0·1	2·4	0	2·1

Incorporation of radioactive phosphate into the nucleotides of PNA

Brain slices were incubated in the bicarbonate buffer containing both glucose and ^{32}P either with or without the addition of cyanide (0·03 M). The nucleotides of the PNA obtained by the method of Hammarsten (1947) were separated chromatographically. Fig. 1 shows the chromatogram and the radioautograph obtained with solvent 1. This

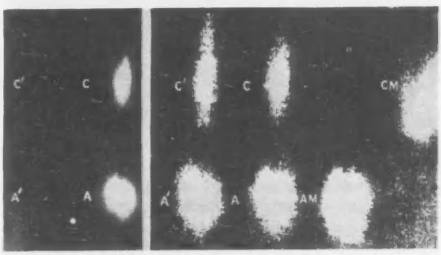

Fig. 1. Chromatogram (solvent 1) of nucleotides of PNA of slices of cat brain incubated in bicarbonate buffer containing glucose and ^{32}P. Radioautograph of the same chromatogram is shown on the left. The spots are: C, cytidylic acid; A, adenylic acid; C′ and A′, cytidylic acid and adenylic acid from PNA of slices incubated in the presence of cyanide (0·03 M); CM, cytidylic acid marker, AM, adenylic acid marker.

solvent separated cytidylic acid (C, C′) and adenylic acid (A, A′) from the other nucleotides. To the right can be seen the separation of the nucleotides. C represents the cytidylic acid and A the adenylic acid of the PNA of the slices incubated without the addition of cyanide. C′ and A′ represent the cytidylic acid and adenylic acid of the slices incubated in a medium to which cyanide had been added. To the left is the radioautograph of the same

chromatogram. The cytidylic acid (C) and adenylic acid (A) from the slices incubated without cyanide darkened the X-ray film, whereas the same nucleotides (C′, A′) from the slices incubated in the presence of cyanide produced no darkening. In the presence of the metabolic inhibitor no ^{32}P was incorporated into the PNA of the slice.

Fig. 2 shows the results obtained with solvent 2. This solvent separated uridylic acid (U, U′) from the other nucleotides. Again it can be seen from the radioautograph, shown on the left, that for slices incubated in the absence of cyanide the ^{32}P was incorporated into the uridylic acid (U) and also into the mixture of adenylic, guanylic and cytidylic acids represented by the spot AGC. When the slices were incubated in the presence of cyanide the incorporation was abolished (U′, AGC′).

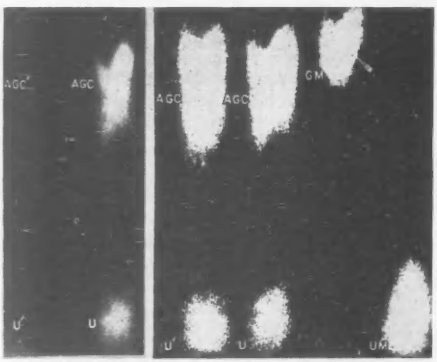

Fig. 2. Chromatogram (solvent 2) of nucleotides of PNA of slices of cat brain incubated in bicarbonate buffer containing glucose and ^{32}P. Radioautograph of the same chromatogram is shown on the left. The spots are: U, uridylic acid; AGC, mixture of adenylic, guanylic and cytidylic acids; U′ is the uridylic acid and AGC′ the mixture of nucleotides from the PNA of slices incubated in the presence of cyanide (0·03 M); GM, guanylic acid marker; UM, uridylic acid marker.

Fig. 3 shows similar data for solvent 3. This solvent system, as described by Markham & Smith (1951), separated adenylic acid *a* (AA), adenylic acid *b* (AB), guanylic acid *a* (GA) and guanylic acid *b* (GB) from the other nucleotides. Again it can be seen from the radioautograph that for the slices incubated in the absence of cyanide there was an incorporation of ^{32}P into all four nucleotides. Cyanide inhibited this incorporation. Fig. 3 also shows the starting point of the chromatogram (D, D′). The intense ultraviolet absorption of these spots is due to the fact that they contain all the DNA, which was not moved in this solvent system. The darkening of the X-ray film by spot D, seen in the radioautograph to the left, does not necessarily mean that ^{32}P was incorporated into the DNA. The darkening was probably caused by traces of much more active contaminants, possibly unhydrolysed PNA, that were not moved on the chromatogram. In obtaining the figures for DNA reported in Table 2, the DNA was precipitated from the solution and thoroughly washed. Nevertheless, contaminating substances

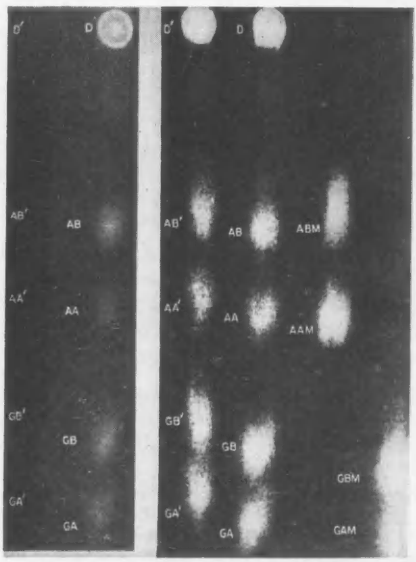

Fig. 3. Chromatogram (solvent 3) of nucleotides of slices of cat brain incubated in bicarbonate buffer containing glucose and ^{32}P. Radioautograph of the same chromatogram is shown on the left. The spots are: D, deoxypentosenucleic acid; AB, adenylic acid *b*, AA, adenylic acid *a*; GB, guanylic acid *b*, GA, guanylic acid *a*; AB′, AA′, GB′, GA′, the same nucleotides from the PNA of slices incubated in the presence of cyanide (0·03 M); ABM, adenylic acid *b* from marker; AAM, adenylic acid *a* from marker; GBM, guanylic acid *b* from marker; GAM, guanylic acid *a* from marker.

may be responsible for the radioactivity of the DNA fraction obtained by the Hammarsten (1947) procedure. It is possible that no ^{32}P was incorporated into the DNA. Fig. 3 also shows that for slices incubated in the presence of cyanide there was no incorporation of ^{32}P into spot D′.

In Table 3 are reported the specific activities of the eluted nucleotides for three experiments. For the slices incubated in the presence of cyanide the specific activities were all very low as was to be anticipated from their failure to darken the X-ray film. When cyanide was absent from the incubating

Table 3. *Specific activities of the nucleotides of the PNA of slices of cat brain incubated at 37·2° in Krebs-Ringer bicarbonate containing* ^{32}P. *Glucose, 0·011 M. Incubation time, 4 hr.*

(Counts/min./μg. P.)

	Expt. 1		Expt. 2		Expt. 3	
	Cyanide (0·03 M)	No cyanide	Cyanide (0·03 M)	No cyanide	Cyanide (0·03 M)	No cyanide
Adenylic acid	1·5	41·7	0·8	35·8	0	50·0
Adenylic acid *b*	1·5	42·9	0	24·4	4·2	48·1
Adenylic acid *a*	2·5	37·1	—	26·4	0	55·8
Guanylic acid *b*	0·9	26·3	0·8	17·3	0·5	35·0
Guanylic acid *a*	1·4	26·5	1·3	14·0	0·4	38·6
Cytidylic acid	1·1	27·4	1·7	25·0	0	30·4
Uridylic acid	2·7	42·6	0	36·8	1·8	35·5
PNA	1·0	29·6	0·6	25·1	1·7	33·9

medium there was an appreciable incorporation of ^{32}P into all of the nucleotides. The specific activities of the individual nucleotides appeared to range around that found for the phosphorus of the hydrolysate reported as PNA in Table 3.

Concerning the relative incorporation of ^{32}P into the various nucleotides, little can be said at present. The figures varied somewhat from experiment to experiment. For purine nucleotides, adenylic acid was always found to be more active than guanylic acid, and for the pyrimidine nucleotides, uridylic acid was more active than cytidylic acid. These findings are similar to those reported by Volkin & Carter (1951) and Davidson & Smellie (1952*b*) for the specific activities of the nucleotides of the PNA of liver tissue after the administration of ^{32}P *in vivo*.

The small differences between the *a* and *b* isomers of adenylic and guanylic acids are not regarded as significant.

Nucleotides of brain PNA

Table 4 shows the molar ratios of the four nucleotides of brain PNA. The figures represent the means from twelve different brain samples. The standard errors give an indication of the significance that may be attached to these figures. Recoveries of total nucleotide (as phosphorus) were of the order of 90–95 %. It will be noted that, on a molar basis, brain PNA contains more guanylic acid than adenylic acid and more cytidylic acid than uridylic acid. It may be more than coincidence that the molar ratios of the four nucleotides are almost the inverse of the ratio of the specific activities, guanylic acid occurring in higher concentration than adenylic acid and having a lower specific activity. Cytidylic acid occurs in higher concentration than uridylic acid and also has a lower specific activity.

As far as we are aware, there are no previous reports in the literature on the distribution of nucleotides in brain PNA. The data presented in Table 2 are similar to those reported by Chargaff *et al.* (1950) for PNA of mammalian liver, with the exception that the proportion of uridylic acid is greater. The figures more closely resemble those reported by Marshak (1951) for the PNA of calf liver and Davidson & Smellie (1952*a*) for the PNA of rat liver.

Table 4. *Molar ratios of the nucleotides in the hydrolysate of PNA of cat brain*

(Mean ± S.E.M. of 12 samples. Adenylic acid = 1.)

Adenylic acid	1·00
Adenylic acid *b*	0·59 ± 0·01
Adenylic acid *a*	0·41 ± 0·01
Guanylic acid *b*	0·82 ± 0·03
Guanylic acid *a*	0·64 ± 0·01
Guanylic acid (by addition)	1·47 ± 0·03
Cytidylic acid	1·20 ± 0·05
Uridylic acid	0·95 ± 0·04

For adenylic acid and guanylic acid, the *b* isomer accounted for 59 and 56% of the total respectively. This value is in agreement with the figures of 50–65 % reported by Carter (1950), Cohn (1951) and Marrian *et al.* (1951) for the nucleotides of yeast PNA, and with the similar figures reported by Marrian *et al.* (1951) for the nucleotides of PNA from the mixed organs of the rat.

DISCUSSION

It should be pointed out that in these experiments only a small percentage of the ^{32}P of the medium was incorporated into the PNA of the slice. Much more was incorporated into the phospholipid, 'phosphoprotein', and the residual ester phosphorus, i.e. the organic phosphorus remaining after the complete extraction of the nucleic acids. After an incubation period of 4 hr. the specific activity of the PNA was of the order of one-thousandth of that of the inorganic phosphate originally in the medium. For tissues other than brain, and for incubation times longer than 4 hr., the incorporation of ^{32}P into PNA was much greater. During the period of incubation there was no demonstrable increase in the amount of PNA present in the slice.

For studies involving the *in vitro* incorporation of ^{32}P into the nucleic acids of tissue slices, a complete separation of the nucleotides is desirable. It has been shown that the isolation procedure of Hammarsten (1947) is satisfactory for PNA, since the specific activity of the PNA so isolated fell within the range observed for the individual nucleotides. The experiments of Davidson & Smellie (1952*b*) would suggest that their shortened partial isolation method (their procedure 2) would be equally satisfactory. However, both methods suffer from the disadvantage that they are not quantitative. The experiments reported here do not indicate whether the Hammarsten procedure is satisfactory for DNA, or whether there is indeed any incorporation of ^{32}P into DNA *in vitro*. The activity of the DNA obtained by the Hammarsten method may be due to contaminating traces of other active substances, possibly traces of PNA not hydrolysed by the alkali. Unequivocal incorporation of ^{32}P into DNA could best be demonstrated by the isolation of the constituent deoxynucleotides, a procedure that would require the enzymic hydrolysis of the DNA. It is clear that for both DNA and PNA the method of Schmidt & Thannhauser (1945) is unsatisfactory, especially for tissue from the nervous system where so much of the protein-bound phosphorus is non-nucleic acid phosphorus (Logan *et al.* 1952).

Previous *in vitro* experiments in which the Schmidt & Thannhauser (1945) method was used, such as those of Mann & Gruschow (1949) with tissue slices, those of Rafelson, Winzler & Pearson (1949) and Rafelson, Pearson & Winzler (1951) with brain mince, and those of Friedkin & Lehninger (1949) with particles from rat liver should be interpreted with caution, although they may show a general trend. The preliminary note of Hokin (1952), who studied slices of pancreas, and the findings of Findlay (1953) in this laboratory would indicate that ^{32}P is readily incorporated into the PNA of slices of a wide variety of tissues.

Objections similar to those made to *in vitro* experiments also apply to many studies in which ^{32}P was administered to animals *in vivo*. The results reported here, taken in conjunction with the findings of Logan *et al.* (1952) and Strickland (1952), suggest that the specific activities of the 'nucleoprotein P' of Dawson & Richter (1950) and the 'residual P' of Bodian & Dziewiatkowski (1950) were greater than the true specific activity of the PNA, and that they were considerably greater than the specific activity of the total nucleic acid, for the DNA is relatively inactive. Similar high specific activities were obtained for the 'nucleic acid' fraction in studies such as those of Erickson, Larson & Gordon (1949), Samuels, Boyarsky, Gerard, Libet & Brust (1951) and Stern & Marshall (1951) in which the TCA extraction procedure of Schneider (1945) was applied to tissue of the nervous system. Experiments have shown that the TCA removes much non-nucleic acid phosphorus (Logan *et al.* 1952) and that this phosphorus has a specific activity higher than that of the PNA and much higher than that of the DNA.

The nucleotides of PNA may be separated by paper chromatography with the use of appropriate solvents. Unfortunately, no one solvent system was found that gave a complete separation of the nucleotides on the same chromatogram. In this regard the methods of ionophoresis developed by Davidson & Smellie (1952*a*) and Markham & Smith (1952) should prove of great value. Magasanik *et al.* (1950), Carter (1950), Marshak & Vogel (1950) and Markham & Smith (1951) achieved a degree of separation, but in each instance the spots for two or more of the nucleotides overlapped to such an extent that specific activity measurements were impossible. Boulanger & Montreuil (1951) reported good separation with systems containing phenol, but under such conditions localization of the spots is difficult.

As reported by Markham & Smith (1951), solvent 3 separated the *a* and *b* isomers of adenylic acid and guanylic acid. These isomers, obtained by the alkaline hydrolysis of PNA, were first reported in hydrolysates of yeast PNA by Carter & Cohn (1949), Carter (1950) and Cohn (1950). Adenylic acids *a* and *b* have been separated one from the other chemically (Riechard, Takenaka & Loring, 1952) and have been synthesized (Brown & Todd, 1952*a*). Adenylic acids *a* and *b* are adenosine-2′-phosphate and adenosine-3′-phosphate, but not necessarily in that order. The finding that the specific activities of the *a* and *b* isomers of either adenylic acid or guanylic acid did not differ significantly from each other, whereas adenylic acid, for example, differed significantly from guanylic acid, is in accord with present concepts of PNA structure and hydrolysis. Brown & Todd (1952*b*) postulated that on alkaline hydrolysis the polynucleotide gives rise to a mixture of the *a* and *b* isomers of each of the mononucleotides by way of an intermediate cyclic 2′:3′-phosphate. Added support is given to this suggestion by the finding of Markham & Smith (1952) that all four cyclic nucleotides (2′:3′-phosphates) were present in both alkaline hydrolysates and ribonuclease digests of yeast PNA and turnip yellow mosaic virus PNA. The natural cyclic nucleotides were found to be indistinguishable chromatographically from the corresponding synthetic nucleotides prepared by Brown, Magrath & Todd (1952). If the theory of Brown & Todd (1952*b*) concerning the origin of the *a* and *b* nucleotides is correct, the similar specific activities of the *a* and *b* isomers of adenylic and guanylic acids would be expected. In experiments in which tracer substances were administered *in*

vivo, the *a* and *b* isomers of adenylic and guanylic acid were shown to be metabolically indistinguishable (Marrian *et al.* 1951, for ^{14}C- and ^{15}N-labelled adenine; Volkin & Carter, 1951, for ^{32}P).

The experiments reported above indicate that ^{32}P is readily incorporated into the PNA of brain slices and that the incorporation is dependent upon the metabolism of the slice. Findlay, Rossiter & Strickland (1953), demonstrate in the following paper that the incorporation is probably related to an active phosphorylating mechanism. The finding that the addition of cyanide to the incubating medium inhibited the incorporation indicates that the separation procedure was satisfactory, for in these experiments all the manipulations were carried out in the presence of concentrations of ^{32}P similar to those used when the cyanide was omitted. As an additional check a small quantity of ^{32}P was added to a mixture of the inert nucleotides and the nucleotides were separated by the usual chromatographic methods. No radioactivity was detected in the spots corresponding to each of the nucleotides.

The active metabolism of brain PNA *in vitro* is in sharp contast to the results of early experiments in which ^{32}P was administered *in vivo* (summarized by Hevesy, 1947, 1948). These showed that little ^{32}P was incorporated into the nucleic acids of brain, suggesting that both the PNA and the DNA of brain were rather inert metabolically. However, as Bakay & Lindberg (1949) and Lindberg & Ernster (1950) have demonstrated, when ^{32}P is injected into the cerebro-spinal fluid it is rapidly taken up by the brain. This ^{32}P is readily incorporated into the PNA and phospholipid fractions (Strickland, 1952). Thus experiments carried out both *in vivo* and *in vitro* have shown that ^{32}P can be rapidly incorporated into the PNA of brain. It follows that the active metabolism of PNA in the nervous system postulated by Caspersson (1947) and Hydén (1943, 1947) may be possible.

SUMMARY

1. Radioactive phosphate is incorporated into the pentosenucleic acid of cat-brain slices respiring in a Krebs-Ringer bicarbonate medium containing glucose and ^{32}P. This was demonstrated by measuring the specific activity of the constituent nucleotides isolated by paper chromatography.

2. The incorporation is abolished by the addition of cyanide to the medium.

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REFERENCES

Bakay, L. & Lindberg, O. (1949). *Acta physiol. scand.* **17**, 179.
Bodian, D. & Dziewiatkowski, D. (1950). *J. cell. comp. Physiol.* **35**, 155.
Boulanger, P. & Montreuil, J. (1951). *Bull. Soc. Chim. biol., Paris*, **33**, 784.
Brown, D. M., Magrath, D. L. & Todd, A. R. (1952). *J. chem. Soc.* p. 2708.
Brown, D. M. & Todd, A. R. (1952*a*). *J. chem. Soc.* p. 44.
Brown, D. M. & Todd, A. R. (1952*b*). *J. chem. Soc.* p. 52.
Carter, C. E. (1950). *J. Amer. chem. Soc.* **72**, 1466.
Carter, C. E. & Cohn, W. E. (1949). *Fed. Proc.* **8**, 190.
Caspersson, T. (1947). *Symp. Soc. exp. Biol.* **1**, 127.
Chargaff, E., Magasanik, B., Vischer, E., Green, C., Doniger, R. & Elson, D. (1950). *J. biol. Chem.* **186**, 51.
Cohn, W. E. (1950). *J. Amer. chem. Soc.* **72**, 1471.
Cohn, W. E. (1951). *J. cell. comp. Physiol.* **38**, *Suppl.* **1**, 21.
Davidson, J. N., Frazer, S. C. & Hutchinson, W. C. (1951). *Biochem. J.* **49**, 311.
Davidson, J. N. & Smellie, R. M. S. (1952*a*). *Biochem. J.* **52**, 594.
Davidson, J. N. & Smellie, R. M. S. (1952*b*). *Biochem. J.* **52**, 599.
Dawson, R. M. C. & Richter, D. (1950). *Proc. Roy. Soc.* B, **137**, 252.
Erickson, T. C., Larson, F. & Gordon, E. S. (1949). *J. Lab. clin. Med.* **34**, 587.
Ernster, L., Zetterström, R. & Lindberg, O. (1950). *Acta chem. scand.* **4**, 942.
Findlay, J. M. (1953). To be published.
Findlay, J. M., Rossiter, R. J. & Strickland, K. P. (1953). *Biochem. J.* **55**, 200.
Folch, J. & Le Baron, F. N. (1951). *Fed. Proc.* **10**, 183.
Friedkin, M. & Lehninger, A. L. (1949). *J. biol. Chem.* **177**, 775.
Hammarsten, E. (1947). *Acta med. scand.* **128**, *Suppl.* **196**, 634.
Hevesy, G. (1947). *Advanc. Enzymol.* **7**, 111.
Hevesy, G. (1948). *Advanc. biol. med. Phys.* **1**, 409.
Hokin, L. E. (1952). *Biochim. biophys. Acta*, **8**, 225.
Holiday, E. R. (1930). *Biochem. J.* **24**, 619.
Hydén, H. (1943). *Acta physiol. scand.* **6**, *Suppl.* 17.
Hydén, H. (1947). *Symp. Soc. exp. Biol.* **1**, 152.
Jeener, R. (1949). *Bull. Soc. Chim. biol., Paris*, **31**, 731.
Lindberg, O. & Ernster, L. (1950). *Biochem. J.* **46**, 43.
Logan, J. E., Mannell, W. A. & Rossiter, R. J. (1952). *Biochem. J.* **51**, 470.
Magasanik, B., Vischer, E., Doniger, R., Elson, D. & Chargaff, E. (1950). *J. biol. Chem.* **186**, 37.
Mann, W. & Gruschow, J. (1949). *Proc. Soc. exp. Biol., N.Y.*, **71**, 658.
Markham, R. & Smith, J. D. (1949). *Biochem. J.* **45**, 294.
Markham, R. & Smith, J. D. (1951). *Biochem. J.* **49**, 401.
Markham, R. & Smith, J. D. (1952). *Biochem. J.* **52**, 552.
Marrian, D. H., Spicer, V. L., Balis, M. E. & Brown, G. B. (1951). *J. biol. Chem.* **189**, 533.
Marshak, A. (1951). *J. biol. Chem.* **189**, 607.
Marshak, A. & Vogel, H. J. (1950). *J. cell. comp. Physiol.* **36**, 97.
Ploeser, J. M. & Loring, H. S. (1949). *J. biol. Chem.* **178**, 431.

Rafelson, M. E., Pearson, H. E. & Winzler, R. J. (1951). *Proc. Soc. exp. Biol., N.Y.*, **76**, 689.
Rafelson, M. E., Winzler, R. J. & Pearson, H. E. (1949). *J. biol. Chem.* **181**, 583.
Riechard, P., Takenaka, Y. & Loring, H. S. (1952). *J. biol. Chem.* **198**, 599.
Samuels, A. J., Boyarsky, L. L., Gerard, R. W., Libet, B. & Brust, M. (1951). *Amer. J. Physiol.* **164**, 1.
Schmidt, G. & Thannhauser, S. J. (1945). *J. biol. Chem.* **161**, 83.
Schneider, W. C. (1945). *J. biol. Chem.* **161**, 293.
Smith, J. D. & Markham, R. (1950). *Biochem. J.* **46**, 509.
Stern, W. E. & Marshall, C. (1951). *Proc. Soc. exp. Biol., N.Y.*, **78**, 16.
Strickland, K. P. (1952). *Canad. J. Res.* (*Med. Sci.*) **30**, 484.
Strickland, K. P., DeLuca, H. A. & Rossiter, R. J. (1953). *Fed. Proc.* (in the Press).
Szafarz, D. & Paternotte, C. (1951). *Bull. Soc. Chim. biol., Paris*, **33**, 1518.
Volkin, E. & Carter, C. E. (1951). *J. Amer. chem. Soc.* **73**, 1519.

Factors Affecting the Incorporation of Radioactive Phosphate into the Pentosenucleic Acids of Brain Slices

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In the preceding paper (DeLuca, Rossiter & Strickland, 1953) it was shown that radioactive phosphate (^{32}P) was incorporated into the pentosenucleic acid (PNA) of cat-brain slices respiring in a glucose-containing Krebs-Ringer bicarbonate solution. Factors affecting this incorporation are now described in more detail. Evidence is presented that the incorporation is dependent upon an active phosphorylating mechanism within the tissue slice.

METHODS

The methods were essentially those described in the preceding paper (DeLuca *et al.* 1953). Where necessary, glucose was omitted from the incubating medium, or replaced by other substrates. All substrates and inhibitors were made up in the Krebs-Ringer bicarbonate and, if necessary, the solution was titrated to the pH of the buffer with HCl or NaOH. For the anaerobic experiments N_2 replaced O_2 in the gas mixture and the gas mixture was passed over heated Cu filings to remove the last traces of O_2.

The nucleic acids were extracted from the tissue and purified by the method of Hammarsten (1947). The specific activity of the PNA nucleotides was determined after two magnesia treatments. The mixed nucleotides so obtained have a specific activity within the range of that given by the individual nucleotides separated chromatographically (De Luca *et al.* 1953). All experiments were done in duplicate. The results reported have been corrected for decay and calculated on the basis of a standard count of $8{\cdot}1 \times 10^5$/min./ml. incubating medium.

RESULTS

Glucose as substrate

Table 1 shows the incorporation of ^{32}P into the PNA of the brain slices. The figures reported for 0 hr. represent slices that were placed in the medium containing the ^{32}P. The flask was shaken to ensure thorough mixing and the entire contents, including the ^{32}P, were then dispersed at 0° in trichloroacetic acid as described for the incubated slices (DeLuca, *et al.* 1953). It can be seen from Table 1 that when glucose, which is well known to increase the oxygen use of brain slices, was omitted from the medium there was a decreased incorporation of ^{32}P into the PNA. The stimulatory effect of glucose became greater as the concentration was increased from 0 to 100 mg./100 ml. Glucose concentrations as great as 800 mg./100 ml. were inhibitory. These experiments suggest a relation between the incorporation of ^{32}P into the PNA and the carbohydrate metabolism of the slice.

Table 1. *Specific activity of PNA of slices of cat brain*

(Slices incubated at 37·2° in Krebs-Ringer bicarbonate containing ^{32}P. Glucose, 0·011 M. Counts/min/μg. P.)

	Expt. 1	Expt. 2	Expt. 3
0 hr.	0·11	0·09	0·08
4 hr.	18·2	26·8	25·8
4 hr. glucose omitted	13·7	18·5	21·3

Fig. 1 shows the time course of the incorporation. After a slight initial lag the incorporation was linear with time for the first 4 hr. Other experiments with longer incubation times confirmed this result and showed that during the period 4 to 6 hr. the incorporation was faster. Since no special precautions were taken to prevent bacterial contamination, these experiments are not reported in detail.

The incorporation of ^{32}P into PNA was greatly suppressed when the conditions were anaerobic

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(Table 2). The incorporation was also much less when the cellular structure of the slice was destroyed by dispersing the tissue in a Potter-Elvehjem type homogenizer (Table 3). The medium used for the preparation of the suspension was the same as that used for the slice experiments, with the exception that nicotinamide was added to inhibit the destruction of cozymase. The suspension was reinforced with adenosine triphosphate (ATP) and cozymase. Fluoride was added to prevent the destruction of the ATP. Each of these additions slightly increased the incorporation of ^{32}P into the PNA, but despite this, the incorporation with the suspension was very poor compared with that obtained with slices (cf. Table 1). The observation that there was at least some incorporation with suspensions would suggest that further experiments with actively respiring suspensions might yield a system capable of incorporating ^{32}P into PNA.

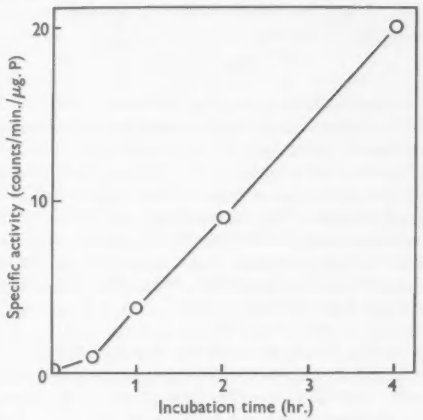

Fig. 1. Specific activity of the PNA of slices of cat brain incubated at 37·2° in Krebs-Ringer bicarbonate. Glucose, 0·011 M.

Table 2. *Effect of anaerobic conditions on the specific activity of the PNA of slices of cat brain*

(Slices incubated at 37·2° in Krebs-Ringer bicarbonate containing ^{32}P, glucose, 0·011 M. Incubation time, 4 hr. Counts/min./μg. P.)

Gas phase	Expt. 1	Expt. 2
95% O_2–5% CO_2	21·1	28·9
95% N_2–5% CO_2	2·1	2·7

Table 3. *Specific activity of PNA of suspensions of cat brain*

(Suspension incubated at 37·2° in Krebs-Ringer bicarbonate containing ^{32}P. Glucose, 0·011 M; cozymase, 0·004 M; ATP, 0·004 M; sodium fluoride, 0·025 M. The brain was dispersed in Ringer containing 0·04 M-nicotinamide. Incubation time, 4 hr. Counts/min./μg. P.)

	Expt. 1	Expt. 2	Expt. 3
0 hr.	1·32	—	0·25
4 hr.	1·95	0·82	0·87

Other substrates

In Table 4 the effect of a number of monosaccharides is shown. Mannose gave an incorporation comparable with that obtained with glucose, but fructose and galactose were generally more variable and less effective. These results were somewhat surprising for, although mannose is known to increase the oxygen consumption of brain tissue respiring *in vitro*, fructose is reported to be equally active (Loebel, 1925; Quastel & Wheatley, 1932). Galactose also increases brain respiration, but the effect is much smaller (Loebel, 1925; Quastel & Wheatley, 1932).

Table 4. *Effect of monosaccharides on the specific activity of the PNA of slices of cat brain*

(Slices incubated at 37·2° in Krebs-Ringer bicarbonate containing ^{32}P. Incubation time, 4 hr. Counts/min./μg. P.)

	Without substrate	With substrate	Percentage increase
Glucose, 0·011 M	18·0	22·0	22
	13·7	18·2	33
	18·5	26·8	45
	21·3	25·8	21
	17·0	26·6	56
	17·2	26·5	54
Mannose, 0·01 M	21·3	30·0	41
	26·7	28·0	5
	17·2	22·3	30
Fructose, 0·01 M	21·3	24·2	14
	26·7	19·3	−28
	17·2	20·1	17
Galactose, 0·01 M	21·3	28·3	33
	26·7	25·0	−6
	62·5	64·5	3

Table 5. *Effect of pyruvate and lactate on the specific activity of the PNA of cat brain*

(Slices incubated at 37·2° in Krebs-Ringer bicarbonate containing ^{32}P. Incubation time, 4 hr. Counts/min./μg. P.)

	Without substrate	With substrate	Percentage increase
Pyruvate, 0·01 M	13·7	24·9	82
	18·5	22·6	22
Lactate, 0·01 M	17·0	19·7	16
	26·7	31·1	16
	17·2	19·9	16

Table 5 shows that both pyruvate and lactate, substrates that are known to increase the oxygen consumption of brain tissue *in vitro* (Loebel, 1925; Quastel & Wheatley, 1932), increased the incorporation of ^{32}P into the PNA. However, both

succinate and L(+)-glutamate were without effect (Table 6), although each of these substrates is oxidized by brain *in vitro* (Quastel & Wheatley, 1932; Krebs, 1935). Weil-Malherbe (1936) showed that L(+)-glutamate increased the oxygen uptake of brain slices, but that D(−)-glutamate was inhibitory. Table 6 shows that D(−)-glutamate also inhibited the incorporation of ^{32}P into PNA. The tricarboxylic acid cycle intermediates, α-ketoglutarate, citrate and malate, did not support the incorporation.

Table 6. *Effect of other substrates on the specific activity of the PNA of slices of cat brain*

(Slices incubated at 37·2° in Krebs-Ringer bicarbonate containing ^{32}P. Incubation time, 4 hr. Counts/min./μg. P.)

	Without substrate	With substrate	Percentage increase
Succinate, 0·05 M	13·7	14·5	6
	18·5	12·8	−31
L(+)-Glutamate, 0·01 M	13·7	14·6	7
	18·5	18·5	0
	17·0	15·5	−9
	17·0	14·0	−18
D(−)-Glutamate, 0·01 M	17·0	11·9	−30
α-Ketoglutarate, 0·01 M	34·3	30·1	−12
Citrate, 0·01 M	34·3	29·4	−14
Malate, 0·01 M	34·3	30·9	−10

Table 7. *Effect of inhibitors on the specific activity of the PNA of slices of cat brain*

(Slices incubated at 37·2° in Krebs-Ringer bicarbonate containing ^{32}P. Glucose, 0·011 M. Incubation time, 4 hr. Counts/min./μg. P.)

Inhibitor	Without inhibitor	With inhibitor	Percentage inhibition
Cyanide, 3×10^{-2} M	21·1	1·2	94
	25·6	0·7	97
	33·9	1·7	95
Azide, 10^{-2} M	35·5	0·6	98
	28·9	0·4	99
Malononitrile, 10^{-2} M	27·5	0·8	97
	40·0	2·3	94
Chloretone, 4×10^{-3} M	35·5	5·6	84
	28·9	6·1	79
Iodoacetate, 10^{-3} M	42·0	14·0	67
Nembutal (pentobarbitone sodium), 10^{-3} M	35·5	21·3	40
	26·8	13·9	48
2:4-Dinitrophenol			
10^{-3} M	42·0	2·45	94
10^{-3} M	27·5	0·5	98
10^{-4} M	27·5	4·7	83
10^{-5} M	27·5	22·0	20

Inhibitors

It has been shown that the incorporation of ^{32}P into the PNA of brain slices is dependent upon the aerobic metabolism of the slice. When the metabolism of the slice was inhibited by any of a number of metabolic inhibitors, the incorporation was greatly decreased (Table 7). With the exception of the lower concentrations of 2:4-dinitrophenol (DNP), all the inhibitors were used in concentrations that are known to decrease the oxygen consumption of the slice. These experiments provide good evidence that the incorporation is a metabolic phenomenon and not solely a simple exchange reaction.

DISCUSSION

The finding that the incorporation of ^{32}P into the PNA of brain slices was increased in the presence of substrates such as glucose, mannose, lactate, or pyruvate, and inhibited by a wide range of metabolic inhibitors indicates that the incorporation is dependent upon the metabolism of the slice. The absence of oxygen or the fragmentation of the tissue, as in the preparation of a suspension, greatly decreased the incorporation. In this regard, the incorporation of ^{32}P into the PNA of brain slices is similar to other synthetic reactions brought about by brain slices, such as the aerobic formation of acetylcholine (Quastel, Tennenbaum & Wheatley, 1936) and the incorporation of ^{32}P into phospholipids (Fries, Schachner & Chaikoff, 1942; Schachner, Fries & Chaikoff, 1942). In general, the incorporation of labelled precursors into the cholesterol, phospholipids, nucleic acids, or proteins of tissue slices is inhibited by anaerobic conditions or by the destruction of the tissue slice.

Of the substances that failed to increase the incorporation of ^{32}P into PNA, glutamate and succinate are of some interest. Both increase the oxygen uptake of brain slices. As pointed out by Weil-Malherbe (1950, 1952), it is unlikely that L(+)-glutamate is a major energy-yielding substrate for brain tissue. The same is probably true for succinate, for it failed to support the aerobic formation of acetylcholine by brain slices (Quastel *et al.* 1936) and it also failed to support the active transport of glutamate into brain tissue against a concentration gradient (Stern, Eggleston, Hems & Krebs, 1949).

In the concentrations used, most of the inhibitors probably affected the incorporation of ^{32}P into PNA as a result of their inhibition of respiration. Because it is representative of a group of metabolic inhibitors whose mode of action may possibly be different, 2:4-dinitrophenol was studied at lower concentrations. Loomis & Lipmann (1948) reported that low concentrations of DNP inhibited the uptake of inorganic phosphate, but not of oxygen, by washed suspensions from rabbit kidney. Although there is still considerable doubt as to the mechanism whereby DNP exerts its effect (Teply, 1949; Judah, 1951), subsequent work in a number of laboratories with a wide variety of tissue preparations, including brain suspensions (Case & McIlwain, 1951), has

shown that DNP is one of a series of substances that may prevent the formation of energy-rich phosphate compounds without decreasing the oxygen consumption. The incorporation of ^{32}P into the PNA of cat-brain slices was greatly inhibited by DNP in a concentration of 10^{-4} M and was slightly inhibited by a concentration as low as 10^{-5} M. This range of DNP concentrations stimulates the oxygen consumption of brain slices (Peiss & Field, 1948; Tyler, 1950; McIlwain & Gore, 1951).

The evidence indicates that in circumstances where the formation of energy-rich phosphate compounds is inhibited, the incorporation of ^{32}P into PNA is also inhibited. It is possible that it may be necessary for the ^{32}P to be incorporated into some such labile phosphate compound before it can be incorporated into the PNA. Under the appropriate conditions ^{32}P is readily incorporated *in vitro* into the labile phosphate compounds of other tissues (Friedkin & Lehninger, 1949*a*, *b*; Green, Atchley, Nordmann & Teply, 1949; Hummel & Lindberg, 1949).

It is also possible that the incorporation of ^{32}P into the PNA of brain slices may be dependent upon the concentration of energy-rich phosphate donors. McIlwain, Buchel & Cheshire (1951) have shown that slices of guinea-pig brain respiring in glucose are able to form phosphocreatine and that the concentration of this substance is maintained at levels approaching those attained *in vivo*. Adenine polyphosphates are probably similarly maintained (McIlwain, 1952). There is a remarkable similarity between the conditions that adversely affect the incorporation of ^{32}P into the PNA of slices of cat brain and the conditions, studied by McIlwain and his collaborators, that are responsible for the failure of guinea-pig brain slices to maintain their level of phosphocreatine. The absence of oxygen or glucose (McIlwain *et al.* 1951; McIlwain, 1952), replacing the glucose by L(+)-glutamate (McIlwain, 1951, 1952), or succinate (Kratzing, 1952), or the presence of a wide range of metabolic inhibitors (Buchel & McIlwain, 1950), is responsible for the failure of both guinea-pig brain slices to maintain the concentration of phosphocreatine and cat-brain slices to incorporate ^{32}P into PNA. Dinitrophenol, which may affect the pattern of brain metabolism differently (McIlwain & Gore, 1951; McIlwain, 1952), also inhibits creatine phosphate formation and the incorporation of ^{32}P into PNA.

SUMMARY

1. The incorporation of radioactive phosphate into the pentosenucleic acid of cat-brain slices respiring in a Krebs-Ringer bicarbonate medium was increased by the addition of glucose or mannose, but not by the addition of fructose or galactose, to the medium.

2. The incorporation of ^{32}P into the pentosenucleic acid was decreased greatly by rendering the conditions anaerobic, or by dispersing the tissue.

3. Pyruvate or lactate supported the incorporation of ^{32}P into the pentosenucleic acid, but not succinate, L(+)-glutamate, D(−)-glutamate, α-ketoglutarate, citrate or L-malate.

4. A wide range of metabolic inhibitors that depress the oxygen consumption of brain slices inhibited the incorporation of ^{32}P into the pentosenucleic acid.

5. 2:4-Dinitrophenol in a concentration that does not decrease the oxygen consumption of brain slices decreased the incorporation of ^{32}P into the pentosenucleic acid.

6. The similarity between the conditions that adversely affect the incorporation of ^{32}P into the pentosenucleic acid of brain slices and those that cause a failure of brain slices to maintain their concentration of phosphocreatine is commented upon.

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REFERENCES

Buchel, L. & McIlwain, H. (1950). *Brit. J. Pharmacol.* **5**, 465.
Case, E. M. & McIlwain, H. (1951). *Biochem. J.* **48**, 1.
DeLuca, H. A., Rossiter, R. J. & Strickland, K. P. (1953). *Biochem. J.* **55**, 193.
Friedkin, M. & Lehninger, A. L. (1949*a*). *J. biol. Chem.* **177**, 775.
Friedkin, M. & Lehninger, A. L. (1949*b*). *J. biol. Chem.* **178**, 611.
Fries, B. A., Schachner, H. & Chaikoff, I. L. (1942). *J. biol. Chem.* **144**, 59.
Green, D. E., Atchley, W. A., Nordmann, J. & Teply, L. J. (1949). *Arch. Biochem.* **24**, 359.
Hammarsten, E. (1947). *Acta med. scand.* **128**, *Suppl.* 196, 634.
Hummel, J. P. & Lindberg, O. (1949). *J. biol. Chem.* **180**, 1.
Judah, J. D. (1951). *Biochem. J.* **49**, 271.
Kratzing, C. C. (1952). Quoted by McIlwain, H. (1952). *Symp. biochem. Soc.* **8**, 27.
Krebs, H. A. (1935). *Biochem. J.* **29**, 1620.
Loebel, R. O. (1925). *Biochem. Z.* **161**, 219.
Loomis, W. F. & Lipmann, F. (1948). *J. biol. Chem.* **173**, 807.
McIlwain, H. (1951). *J. ment. Sci.* **97**, 674.
McIlwain, H. (1952). *Biochem. J.* **52**, 289.
McIlwain, H., Buchel, L. & Cheshire, J. D. (1951). *Biochem. J.* **48**, 12.

McIlwain, H. & Gore, M. B. R. (1951). *Biochem. J.* **50**, 24.
Peiss, C. N. & Field, J. (1948). *J. biol. Chem.* **175**, 49.
Quastel, J. H., Tennenbaum, M. & Wheatley, A. H. M. (1936). *Biochem. J.* **30**, 1668.
Quastel, J. H. & Wheatley, A. H. M. (1932). *Biochem. J.* **26**, 725.
Schachner, H., Fries, B. A. & Chaikoff, I. L. (1942). *J. biol. Chem.* **146**, 95.
Stern, J. R., Eggleston, L. V., Hems, R. & Krebs, H. A. (1949). *Biochem. J.* **44**, 410.
Teply, L. J. (1949). *Arch. Biochem.* **24**, 383.
Tyler, D. B. (1950). *J. biol. Chem.* **184**, 711.
Weil-Malherbe, H. (1936). *Biochem. J.* **30**, 665.
Weil-Malherbe, H. (1950). *Physiol. Rev.* **30**, 549.
Weil-Malherbe, H. (1952). *Symp. biochem. Soc.* **8**, 16.

Effect of Starvation on the Composition of the Liver Cell

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It has long been known that starvation causes the liver as a whole to shrink, but it is only with the development of analytical and histological techniques during the last 100 years that this reduction in size has been attributed to a decrease in the size of the individual liver cells, caused by a rapid loss of glycogen and a slow but persistent loss of protein and water. Numerous descriptions of the changes produced by fasting in the livers of many species have appeared and many authorities have discussed and reviewed them (Benedict, 1915; Kosterlitz & Campbell, 1945; Peters & Van Slyke, 1946). Most workers were interested principally in the organic matter of the liver (Davidson, 1947; Davidson & Waymouth, 1944, 1946; Dible, 1932; Addis, Poo & Lew, 1936) or, more recently, in its enzymes (Miller, 1948; Lightbody & Kleinman, 1940), and few paid attention to the minerals. Moreover, no one has analysed the changes which take place in the liver cell itself, although Elman, Smith & Sachar (1943) and Kosterlitz (1947) correlated their chemical findings with the histological appearance of the liver.

A method by which the results of a fairly complete chemical analysis of samples of whole liver can be used to deduce the mass and composition of the liver cell was described in detail in a previous paper and is given here in outline (Harrison, 1953*a*). This method is based upon the assumption that the masses of the liver cells, like those of their nuclei, are dependent upon the number of chromosomes which they contain and are therefore related to their content of deoxynucleic acid. Moreover, the average mass of the tetraploid cell, whether it contains the tetraploid number of chromosomes in a single nucleus or is binucleate, is twice that of the diploid cell, containing the diploid number of chromosomes in its nucleus. Similarly, the octoploid cell is four times as large as the diploid cell. It follows that the average mass of the diploid cell, which is independent both of the degree of polyploidy and of the proportion of binucleate cells, may be obtained from the ratio of the amount of deoxynucleic acid (DNA) in a diploid nucleus to that in a unit weight of liver tissue. The amount of DNA in the diploid nucleus was determined in the kidney, a tissue known to contain only diploid nuclei. The average mass of the diploid cell determined in this way was considered a better unit for comparison than the mass of the 'mean liver cell', the average for all the cells in the liver. A correction was made for that portion of the mass of the liver which lies outside the cells, which was assumed to be equal to the mass of water, protein and minerals in the blood, lymph, bile and interstitial fluid, and was deduced from the chloride space of the liver and the composition of the serum. The water, protein and minerals in the extracellular fluids of the liver were subtracted from values for whole tissue to determine the composition of the cells; it was assumed that all of the neutral lipids, glycogen, phospholipids, ribonucleic acid (RNA), DNA, inorganic iron, copper and zinc was intracellular.

This method has now been used to follow the changes in the liver cells of adult male and female rats during a 6-day period of starvation and to define differences in the responses of the two sexes to fasting.

EXPERIMENTAL

Animals. Thirty-five male and thirty-five female black and white hooded rats of the Lister strain were used. The male and female rats were from the same litters and were 3–4 months old. Until the fast began they were fed on a stock diet consisting of bread, milk, whole wheat, yeast, fishmeal, oatflakes and bran. The rats were weighed twice each week for 3 weeks before the fast to ensure that they were growing at a normal rate. The males and females were divided into five comparable groups of seven rats, according to their mean weights on the 3 days before the fast, and to their increase in weight during the three pre-experimental weeks. One group of either sex was killed at the start between 11 a.m. and 12 noon and served as controls. Their food had been removed 2 hr. previously. The remaining rats were kept in cages which were raised to prevent coprophagy.

Water was given *ad lib.* but the animals received no food of any kind. The rats were weighed daily at 11 a.m., and a group of each sex was killed after 1, 2, 4 and 6 days, respectively, at the same time of day as the control group.

Methods of analysis. The rats were stunned and then decapitated; samples of blood were collected from the carotid in tubes without anticoagulant and the serum separated. A small sample was rapidly snipped from the median lobe of the liver for estimating glycogen and water (Harrison, 1953*a*). The remainder of the liver was quickly removed, weighed, cut into small pieces and placed in ice-cold water in the refrigerator. The large samples from the livers of the seven rats were pooled and dispersed at 0° with five times their weight of water (Folley & Watson, 1948); the following constituents were estimated in the suspension as described previously: protein, phospholipids, neutral lipids, RNA, DNA, Cl, total P, inorganic P, total Fe, inorganic Fe, Cu, Zn, Na and K (Harrison, 1953*a*). The serum from the rats' blood was pooled by mixing an equal volume of serum from each rat in the group, and samples were taken for the estimation of protein, Na and Cl.

Analysis of nuclei. The nuclei were separated by the method of Dounce (1943) from the livers of three normal male rats and three normal female rats, and from the livers of three male and three female rats after a fast of 6 days; these animals were in every respect similar to those used in the main experiment. Nuclear suspensions were then prepared for determining the average amount of DNA and protein in the nucleus, and its dry weight (Harrison, 1953*a*).

Binucleate cells. Suspensions of cells were prepared from the livers used for the analysis of the nuclei for determination of the percentage of binucleate cells (Harrison, 1953*b*).

The volume of extracellular water (ECW). This was deduced from the chloride content of the liver and of the serum by the modified Truax (1939) method (Harrison, 1953*a*), assuming that there were 2 ml. blood/100 g. liver before the fast and on the sixth day, and 3 ml. blood/100 g. liver on the first, second and fourth days. These assumptions were based on the results of Kosterlitz (1947) and Truax (1939) for normal livers, and upon the rapid initial fall in the mass of the livers and also of their cell contents. In addition, Elman *et al.* (1943) observed histologically that the sinusoids were wider in the livers of fasted dogs. Even if these assumptions are not justified, the error introduced would be small since a variation of 1 ml. blood/100 g. liver would cause a difference of 1 ml. ECW/100 g. liver. The volume of intracellular water (ICW) was taken as the difference between the volumes of total water and of ECW.

Mass of diploid cell. The portion 'e' of the mass which lies outside the cells was calculated from the volume of ECW (Harrison, 1953*a*). The average mass of the diploid cell,

$$d = \frac{(1-e)\,b}{h},$$

where b is the amount of DNA in the diploid nucleus, and h the amount of DNA in unit weight of liver tissue. The ratio b/h represents the combined mass of the diploid cell and its share of extracellular material.

Mass of diploid nucleus. The average mass of the diploid nucleus was taken as three times the average dried mass of the liver nuclei making suitable correction for the presence of polyploid nuclei (Harrison, 1953*a*).

Composition of the liver cell. Neutral lipids, glycogen, phospholipids, DNA, RNA, inorganic Fe, Cu and Zn were

Table 1. *The composition of the livers of male and female rats during a 6-day period of starvation*

(Standard deviations of the means for a group of seven rats are given in parentheses.)

Sex	Length of fast (days)	Increase in weight during 3 weeks' pre-experimental period (g.)	Body weight		Liver weight (g.)	g./100 g. Fresh liver					
			Initial (g.)	At death (g.)		Water	Protein	Glycogen	Neutral lipids	Phospho-lipids	RNA	DNA
Male	0	34 (14)	270 (24)	270 (24)	8·91 (1·49)	69·3 (0·3)	18·0	4·3 (1·0)	1·5	2·8	0·96	0·21
	1	33 (11)	272 (18)	256 (18)	7·03 (0·38)	69·1 (0·1)	22·2	0·09 (0·09)	1·2	3·1	1·15	0·27
	2	33 (19)	271 (21)	244 (19)	6·70 (0·54)	68·8 (0·6)	21·7	0·07 (0·08)	2·7	3·2	1·14	0·27
	4	33 (18)	271 (22)	229 (18)	5·80 (0·59)	69·1 (0·5)	21·7	0·55 (0·27)	2·4	3·1	1·11	0·31
	6	32 (15)	271 (23)	210 (20)	5·41 (0·58)	68·3 (1·3)	21·4	0·84 (0·31)	2·3	3·3	1·13	0·33
Female	0	12 (7)	208 (16)	208 (18)	6·20 (0·61)	70·6 (0·7)	18·3	4·0 (0·7)	0·6	2·7	1·08	0·26
	1	12 (5)	209 (17)	200 (15)	5·13 (0·50)	70·6 (0·5)	20·6	0·13 (0·09)	1·5	3·1	1·21	0·31
	2	12 (10)	209 (15)	189 (16)	4·67 (0·22)	70·5 (0·7)	21·0	0·07 (0·07)	2·5	3·6	1·25	0·33
	4	12 (7)	208 (15)	180 (15)	4·39 (0·28)	70·2 (1·1)	21·0	0·16 (0·14)	3·5	3·5	1·12	0·35
	6	12 (5)	208 (16)	167 (14)	4·44 (0·04)	69·2 (0·9)	18·9	0·36 (0·35)	5·4	3·2	1·10	0·36

Table 2. *The inorganic constituents in the livers of male and female rats during a 6-day period of starvation*

(Values for groups of seven rats expressed/100 g. liver.)

Sex	Length of fast (days)	Total iron (mg.)	Inorganic iron (mg.)	Copper (mg.)	Zinc (mg.)	Potassium (m-equiv.)	Sodium (m-equiv.)	Chloride (m-equiv.)	Total P (g.)	Inorganic P (mg.)
Male	0	17	13·2	0·42	4·2	8·10	3·30	2·55	0·37	49
	1	19	14·8	0·55	7·0	8·55	4·30	2·74	0·38	54
	2	20	18·3	0·61	6·6	8·10	4·35	3·00	0·39	52
	4	22	19·9	0·61	7·3	8·30	4·25	2·82	0·39	46
	6	25	22·8	0·61	7·7	8·20	4·05	2·69	0·41	45
Female	0	46	44	0·52	3·6	8·67	3·65	2·64	0·36	34
	1	55	53	0·64	6·3	8·90	3·82	2·76	0·39	43
	2	52	50	0·65	7·8	8·90	4·09	2·97	0·41	48
	4	64	63	0·63	—	8·85	3·61	2·71	0·41	45
	6	72	70	0·65	9·9	8·10	3·61	2·74	0·40	40

considered entirely intracellular components; hence the average amount of each of these constituents in a cell was equal to the amount in the combined mass b/h. Allowance was made for extracellular water, protein and minerals in deducing the amount of water, protein and K within the diploid cells.

No attempt has been made to draw smooth curves through the points in any figure. They have been joined by straight lines which are not meant to represent the changes during the intervening periods but merely to indicate general trends.

Table 3. *The concentration of protein, sodium and chloride in the serum of adult male and female rats during starvation*

Sex	Length of fast (days)	Protein (g./100 ml. serum)	Sodium (m-equiv./l. serum)	Chloride (m-equiv./l. serum)
Male	0	7·18	152	103
	1	7·12	160	104
	2	7·00	158	102
	4	6·88	159	103
	6	6·75	157	105
Female	0	7·68	154	105
	1	7·62	160	105
	2	7·55	161	104
	4	7·55	158	106
	6	6·68	156	106

RESULTS

Composition of liver and serum

Tables 1–3 contain the results from the analysis of the liver and serum during the 6-day period of starvation. The values in these tables, together with those illustrated in Fig. 1 for the volume of ECW, have been used to calculate the mass and composition of the diploid liver cell, on the assumption that the amount of DNA in the diploid nucleus was $5{\cdot}7 \times 10^{-6}$ μg. (Harrison, 1953*a*). Fig. 1 sugggests that starvation was accompanied by a rise in the volume of ECW and conversely a fall in that of ICW during the first 48 hr. of the fast, but thereafter until the end of the fast the changes were reversed.

The same proportion of cells (0·3) was binucleate in suspensions of liver cells from the normal male and female rats. Fasting produced no change in this value in either sex and this agreed with the histological appearance of the livers. No evidence of cell

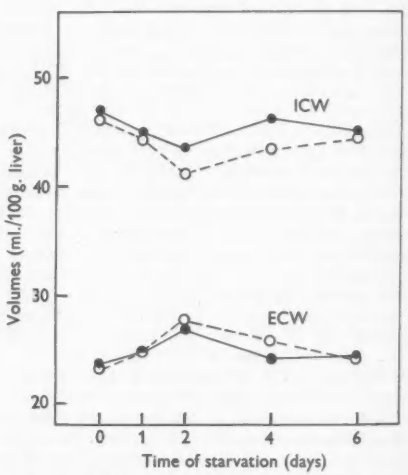

Fig. 1. Volume of ECW, lower curves, and ICW, upper curves, in the livers of male and female rats during a fast of 6 days. ○---○, Male; ●—●, female.

necrosis was observed in sections of the livers after 6 days' fasting; moreover, the total amount of DNA in the liver remained constant throughout the fast ($18{\cdot}3 \pm 0{\cdot}3$ mg. in the male rats, and $15{\cdot}8 \pm 0{\cdot}3$ mg. in the female rats).

Mass of the cell and of its nucleus

Fig. 2 illustrates the changes in the mass of the diploid liver cell. The average mass of the female cell was initially 80% of that of the male cell. During the first 48 hr. the mass of the female cell

Table 4. *The effect of starvation on the average amount of DNA/liver nucleus, and on the mass and 'protein' content of the diploid liver nucleus*

Sex	Length of fast (days)	Body weight: Initial (g.)	Body weight: At death (g.)	Liver weight (g.)	DNA/liver nucleus (10^{-6} μg.)	'Protein'/diploid nucleus (10^{-6} μg.)	Mass of diploid nucleus (10^{-4} μg.)
Male	0	270	270	8·1	9·6	32	1·5
	6	265	204	5·3	9·6	31	1·5
Female	0	205	205	6·5	10·0	27	1·2
	6	210	155	4·3	9·9	27	1·1

decreased to three-quarters of its initial value, and from then until the end of the fast it slowly fell to 73 % of its initial value. The male cell, on the other hand, lost a quarter of its weight in the first 24 hr.; by the end of the fast it had lost 37 %. Thus, although the male liver cell initially had a larger mass than the female cell, their average masses were not very different at the end of the fast (12·8, cf. $11·6 \times 10^{-4}$ μg. respectively). On the other hand, Table 4 shows that the mass of the nucleus was almost the same at the beginning and end of the fast in both sexes. The amounts of DNA and 'protein' in the nucleus were also unchanged by the fast. Whether the average amount of protein found in the nucleus represents the amount present *in vivo* is not certain (Dounce, Tishkoff, Barnett & Freer, 1950; Harrison, 1953*a*), but since the same procedure was always followed in preparing the nuclear suspensions, it is probable that the same proportion of protein was recovered each time. These findings are consistent with the hypothesis of Leuchtenberger & Schrader (1951) that the volume of the nucleus is determined by the amount of protein in it. Furthermore, since there was no change in the size or number of nuclei in the liver and only a small proportionate change in its extracellular phase, such alterations as occurred must have been largely confined to the cytoplasm. The specific gravities of the nucleus and of the cell were assumed to be unaltered by the fast in calculating the nucleo-cytoplasmic ratio (see Harrison, 1953*a*); the ratio increased from 8 % in the normal livers of both sexes to 13 % in the males and 12 % in the females.

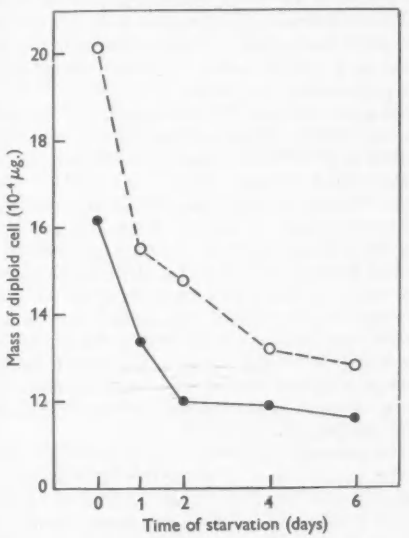

Fig. 2. Mass of the diploid liver cell of male and female rats during a 6-day period of starvation. O---O, Male; ●—●, female.

Composition of the cell

The amounts of each constituent of the cell are listed in Table 5, and the percentage of the cell mass which each contributes is represented in Fig. 3. The greater mass of the normal male cell is due to larger amounts of water, protein, glycogen, neutral lipids and phospholipids. The normal female cell, however, contains a greater percentage of water and this it maintained throughout the experiment. The water in the liver cell fell in the course of the first 24 hr. of the fast by $3·2 \times 10^{-4}$ μg. in the male and $1·9 \times 10^{-4}$ μg. in the female, so that the male cell had lost 1·7 times as much water as the female. The greater loss of water by the male liver cell may have been associated with its larger initial store of glycogen, which was almost completely lost during the first 24 hr. Fenn (1939) has suggested that the breakdown of glycogen was accompanied by a simultaneous loss of water from the cells. More water was lost during the first day while glycogen was disappearing than at any other period of the fast, the amounts lost being equivalent to 25 and 18 % of the initial amounts in the male and female cells; at the end of the fast the water had decreased to 61 and 69 % of the initial values, respectively. These changes caused the percentage of water in the cells to decrease during the first 2 days by a little more than 2 % in the males and a little less in the females; thereafter it tended to rise, but fell again at the end of the fast.

There was a similar percentage of protein in the normal cells of both sexes, and in the course of the first 2 days this increased, although the cells were

Table 5. *The composition of the diploid liver cell of male and female rats during a 6-day period of starvation*

(Results expressed as $\mu g. \times 10^{-4}$.)

Sex	Length of fast (days)	Water	Glycogen	Protein	Neutral lipids	Phospho-lipids	RNA	DNA
Male	0	12·5	1·17	4·35	0·41	0·76	0·26	0·06
	1	9·4	0·02	4·26	0·25	0·66	0·24	0·06
	2	8·7	0·02	4·12	0·57	0·68	0·24	0·06
	4	8·0	0·10	3·58	0·44	0·56	0·20	0·06
	6	7·7	0·15	3·39	0·40	0·57	0·20	0·06
Female	0	10·3	0·87	3·56	0·13	0·59	0·24	0·06
	1	8·4	0·02	3·38	0·27	0·57	0·22	0·06
	2	7·5	0·01	3·18	0·43	0·62	0·21	0·06
	4	7·5	0·03	3·06	0·57	0·57	0·18	0·06
	6	7·1	0·06	2·68	0·85	0·50	0·17	0·06

losing protein. This they did at a fairly constant rate so that on the 6th day the male and female cells contained 75 and 78 % of their initial amounts of protein. The protein in the female cell fell slightly more between the 4th and 6th days than that in the males, and this greater fall coincided with a fall in the concentration of protein in the female serum.

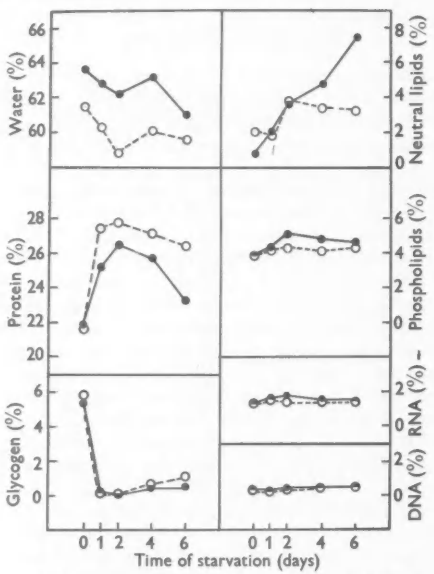

Fig. 3. Percentage composition of the liver cells of male and female rats during a 6-day period of starvation. O---O, Male; ●—●, female.

The metabolism of fats in the cells of the male and female liver appeared to respond to starvation in a slightly different manner. Initially the male cell contained about 3 times as much neutral lipid as the female cell, both values being quite small. In the first day the male cell lost neutral lipids, whereas the female cell gained them, so that at the end of the day both cells contained the same amount. During the following day both cells stored neutral lipids, and this the female cell continued to do at a uniform rate until the end of the fast when neutral lipids made up 7 % of its mass. This store was probably not pathological for there was no sign of fat in histological sections. Furthermore, the value of 7 % was derived from the analytical value of 5·4 g. neutral lipid/100 g. liver, which is less than Glynn, Himsworth & Lindan (1948) found in mildly fatty livers. Meanwhile, the neutral lipids in the male cell fell with its mass, so that from the 2nd to the 6th day there was almost a constant percentage of neutral lipids within it (3 %). During this period the male cell had accumulated twice as much glycogen as the female cell, although both contained very little compared with normal cells. The percentage of phospholipids in the female cell rose distinctly in the first 2 days and thereafter fell again, whereas in the male cell it remained practically unchanged.

The male cell contained 1·1 times as much RNA as the female cell, and during the fast a little more was lost from the female cell. Although RNA is found principally in the cytoplasm the amount present in the cell is apparently not a satisfactory index of the mass of the cytoplasm. In calculating the composition of the cells, it was assumed that the amount of DNA per diploid nucleus was the same in both sexes, and remained constant throughout the fast. This made the percentage of DNA a little higher in the female than in the male cell, and both values increased during the fast.

Minerals in the cell

In Table 6 are listed the amounts of iron, copper and zinc in the liver cells of both sexes, assuming that all the inorganic iron, copper and zinc in the liver were intracellular. The inorganic iron does not represent all the iron located within the cells for they possess enzymes containing haematin, but the iron contributed by these is negligible compared with

Table 6. *The amounts of iron, copper, zinc and potassium in the diploid liver cell of male and female rats during a 6-day period of starvation*

Sex	Length of fast (days)	Iron (10^{-7} μg.)	Copper (10^{-8} μg.)	Zinc (10^{-7} μg.)	Potassium (10^{-9} m-equiv.)
Male	0	3·5	1·1	1·1	2·1
	1	3·1	1·1	1·5	1·7
	2	3·9	1·3	1·4	1·6
	4	3·7	1·1	1·3	1·4
	6	4·1	1·1	1·2	1·3
Female	0	9·6	1·2	0·8	1·8
	1	9·6	1·2	1·2	1·5
	2	8·8	1·1	1·3	1·4
	4	10·0	1·0	—	1·3
	6	10·1	1·0	1·6	1·2

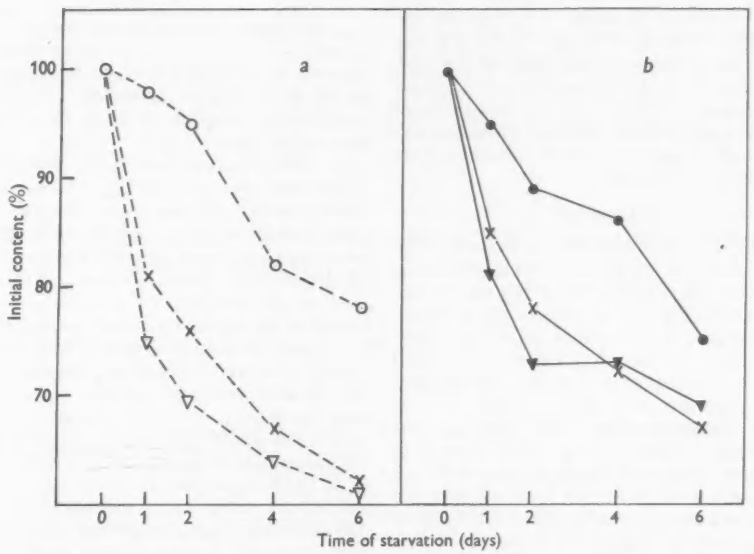

Fig. 4. Percentage of water (▽, ▼), protein (○, ●), and potassium (×) retained in the diploid liver cell after fasting for 1, 2, 4 and 6 days, for the livers of (*a*) male, and (*b*) female rats.

that 'stored' in the liver. The liver cells of the normal male and female rats contained similar amounts of copper and zinc whereas those of the female contained 3 times as much iron as those of the male. Fasting for 6 days produced little change in the 'storage' of these minerals within the cells, although there seemed to be a tendency for zinc to accumulate in the female cell. The copper in the cell was located principally, if not entirely, in the nucleus (Harrison, 1953*a*); at the end of the fast there was about the same amount of copper in the nucleus as at the beginning of the experiment, $0{\cdot}9 \times 10^{-8}$, cf. $0{\cdot}8 \times 10^{-8}$ μg. for the male cell, and $1{\cdot}0 \times 10^{-8}$, cf. $1{\cdot}2 \times 10^{-8}$ μg. for the female cell.

The normal male liver cell contained a little more potassium than the female (see Table 6), and during the fast the potassium fell in the liver cells of both sexes. There was a drop of 19 % in the male cell and of 17 % in the female cell by the end of the first day; thereafter the potassium fell more slowly, but with larger losses between the 4th and 6th days in the female. By the end of the 6th day the liver cells of the male and female rats had lost 38 and 33 % of their initial contents of potassium, and these losses corresponded with their losses of water (39 and 31 % respectively). Fenn (1939) suggested that the disappearance of glycogen from liver cells was associated with the removal of potassium as well as of water, and conversely that the deposition of glycogen was associated with a return of water and potassium. He also found shifts of water with changes in the protein content of the liver, but he made no mention of movements of potassium with these changes. Fig. 4 shows the percentages of

water, protein and potassium which remained in the diploid liver cell at successive stages of the fast; the graphs for the liver cells of the male rats are given on the left-hand side of the figure whereas those for the female rats are on the right. It will be recalled that the glycogen had almost disappeared from the liver cells during the first 2 days of the fast, after which there were small amounts of glycogen, twice as much in the male cell as in the female cell. If the movement of potassium from the liver cell were linked only with the movement of glycogen, then between the 2nd and 4th days the potassium in the cells should have increased slightly. However, this did not occur, and from the 1st to the 6th days the graphs showing losses of protein and potassium are almost parallel. This would indicate that changes in the content of protein were accompanied by comparable changes in that of potassium. It might be a fair summing up to state simply that losses of both glycogen and protein reduced the amount of intracellular fluid, and with it the amount of its principal cation, potassium.

DISCUSSION

The changes in the composition of the cells were practically confined to the cytoplasm and were similar in both sexes during the first 4 days; both cells lost a considerable portion of their water, protein, glycogen, phospholipids and RNA. Thereafter the female cells tended to accumulate neutral lipids, whereas those of the males accumulated glycogen. Glycogen and neutral lipids do not accumulate simultaneously in the liver, and the normal liver accumulates glycogen in preference to neutral lipids. Hence it would appear that the normal functions of the female liver cell were the more disturbed, and this was borne out by the fall in the serum protein during the last 2 days of the fast. The female rats seemed to have reached a more advanced stage in the syndrome of starvation than the males at corresponding times. This was a little surprising for younger rats are thought to have somewhat less resistance to fasting than older ones because of the greater demands of growth. The male rats which were considerably heavier than the females, were a little younger since the experiment on the males was performed 2 weeks before that on the females. Furthermore, the male rats were growing considerably faster, at least until the growth of both was arrested by fasting. The difference between the sexes was however of no great magnitude.

There seemed to be no impairment in the ability of the liver to retain its stores of iron, copper and zinc, for the intracellular stores of these minerals were unchanged in both sexes throughout the fast. Whether they would have been drawn upon or would alternatively have been increased during a longer fast remains undemonstrated. Visible deposits of iron have been found in livers of undernourished persons (Sherlock & Walshe, 1951) and of South African natives with pellagra (Gillman & Gillman, 1945), presumably as a result of breakdown of the muscles and red blood corpuscles. It is doubtful, however, if the changes which occur are comparable with those during fasting, for the changes found in protein deficiency (Kosterlitz, 1947; Elman *et al.* 1943) and after feeding reduced rations (Wertheimer & Ben-Tor, 1950) were not those found in starvation.

SUMMARY

1. A method for the determination of the composition of the liver cells from the results of chemical analysis of samples of whole liver has been employed to investigate the effects of starvation upon the size and composition of the liver cells of male and female rats.

2. Thirty-five male and thirty-five female young adult rats were divided into five similar groups. Seven rats of each sex were killed for analysis of their livers before the commencement of the experiment, and on the first, second, fourth and sixth days of the fast. Similar rats submitted to a precisely similar fast were used for analysis of the liver cell nuclei at the beginning and at the end of the fast.

3. As the animals lost weight, their livers became smaller, but the size and composition of the nuclei did not alter, and neither did the amount of nuclear material in the liver. It follows that the reduction in size of the whole liver was the result of the loss of cytoplasmic constituents from its cells.

4. Glycogen almost disappeared from the liver cells of both sexes during the first 24 hr. Thereafter the amount increased somewhat in the male cell but not in that of the female, which showed instead an increasing amount of neutral lipid until the end of the fast.

5. The cells lost protein throughout the fast, and a somewhat greater loss from the female liver cells during the final 2 days was accompanied by a fall in serum protein.

6. Potassium and water varied with the mass of the cells.

7. The amounts of iron, copper and zinc in the hepatic cells of both sexes were unchanged by the fast.

I wish to thank the University of New Zealand for the Post-graduate Travelling Scholarship in Science during the tenure of which this work was carried out. I also wish to thank Prof. R. A. McCance, F.R.S., for suggesting the original problem from which this work has developed and for his constant encouragement and criticism. I am indebted to Dr A. M. Barrett of the Department of Pathology for preparing and reporting on the sections of the livers, and Miss Pamela Stoakley for valuable technical assistance.

REFERENCES

Addis, T., Poo, L. T. & Lew, W. (1936). *J. biol. Chem.* **115**, 111, 117.
Benedict, F. G. (1915). *A Study of Prolonged Fasting.* Washington, D.C.: Carnegie Institute.
Davidson, J. N. (1947). *Symp. Soc. exp. Biol.* **1**, 77.
Davidson, J. N. & Waymouth, C. (1944). *Nature, Lond.*, **154**, 207.
Davidson, J. N. & Waymouth, C. (1946). *J. Physiol.* **105**, 191.
Dible, J. H. (1932). *J. Path. Bact.* **35**, 451.
Dounce, A. L. (1943). *J. biol. Chem.* **147**, 685.
Dounce, A. L., Tishkoff, G. H., Barnett, S. R. & Freer, R. M. (1950). *J. gen. Physiol.* **33**, 629.
Elman, R., Smith, M. G. & Sachar, L. A. (1943). *Gastroenterology*, **1**, 24.
Fenn, W. O. (1939). *J. biol. Chem.* **128**, 297.
Folley, S. J. & Watson, S. C. (1948). *Biochem. J.* **42**, 204.
Gillman, J. & Gillman, T. (1945). *Arch. Path.* (*Lab. Med.*), **40**, 239.
Glynn, L. E., Himsworth, H. P. & Lindan, O. (1948). *Brit. J. exp. Path.* **29**, 1.
Harrison, M. F. (1953*a*). *Proc. Roy. Soc.* B. **141**, 203.
Harrison, M. F. (1953*b*). *Nature, Lond.*, **171**, 611.
Kosterlitz, H. W. (1947). *J. Physiol.* **106**, 194.
Kosterlitz, H. W. & Campbell, R. M. (1945). *Nutr. Abstr. Rev.* **15**, 1.
Leuchtenberger, C. & Schrader, F. (1951). *Biol. Bull., Wood's Hole*, **101**, 95.
Lightbody, H. D. & Kleinman, A. (1940). *Proc. Soc. exp. Biol., N.Y.*, **45**, 25.
Miller, L. L. (1948). *J. biol. Chem.* **172**, 113.
Peters, J. P. & Van Slyke, D. D. (1946). *Quantitative Clinical Chemistry. Interpretations*, **1**, 2nd ed. London: Baillière, Tindall and Cox.
Sherlock, S. & Walshe, V. M. (1951). *Spec. Rep. Ser. med. Res. Coun., Lond.* no. 275, p. 111.
Truax, F. L. (1939). *Amer. J. Physiol.* **126**, 402.
Wertheimer, E. & Ben-Tor, V. (1950). *Brit. J. Nutr.* **4**, 1.

The Presence of Potassium as a Source of Inaccuracy in the Chemical Estimation of Sodium in Urine

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Most methods for the chemical estimation of sodium in urine are based on precipitation of the metal quantitatively as the triple salt, sodium zinc uranyl acetate. This is subsequently estimated gravimetrically, colorimetrically, or by titration with alkali.

An early study of this method was made by Barber & Kolthoff (1928), and many subsequent modifications have been suggested (McCance & Shipp, 1931; Hoffman & Osgood, 1938; Bradbury, 1946; Albanese & Lein, 1948). In our hands, one version of the method, that of Dreguss (1939), has proved reliable and accurate for simple solutions of a sodium salt and for blood serum.

Difficulties were encountered, however, in applying the method to urine. The apparent sodium content was higher than expected on a basis of equivalence with the chloride, and higher than that obtained by flame photometry (Holiday & Preedy, 1953), especially in urines of low chloride content. This finding was investigated further.

EXPERIMENTAL

The method used was that of Dreguss (1939) with the following minor modifications. The solution was not heated after the addition of ethanolic zinc acetate; it was then centrifuged, not filtered. The volumes of supernatant fluid and of zinc uranyl acetate solution were 2 ml. each, instead of 1·5 ml. The solution in which precipitation took place was left for 3–24 hr. at room temperature. A filter stick with a tuft of asbestos was used for washing the precipitate, instead of repeated centrifuging.

Calculation. From the equation and calculation given by Dreguss, 1·0 ml. 0·1 N-NaOH used in titration is equivalent to 0·256 mg. Na. In our hands, this formula gave accurate results on the standard NaCl solution. (Dreguss used the formula 1·0 ml. 0·1 N-NaOH ≡ 0·243 mg. Na, apparently on account of Na impurities in his samples of zinc uranyl acetate.)

Apparatus for flame spectroscopy. The flame photometer of Holiday & Preedy (1953) was used. The lens and filter system was dismantled, and a Hilger monochromator, Type D. 246, with glass prism, was interposed between the flame source and the image converter. Spectral analysis of the flame emission was carried out by scanning the spectrum between 400 and 800 mμ.

RESULTS

Accuracy of the chemical method

A standard solution of NaCl was prepared, containing 3 mg. Na/ml. Duplicate estimations of Na in this solution were carried out whenever a batch of urines was analysed. The relevant statistics of these

standard estimations and of the duplicate urine estimations are as follows:

Standard NaCl *solution*. Taking the calculated titration figure as 100, the mean of 54 estimations was 99·96 (S.D., 0·20).

Urine. The standard error of difference between 87 pairs of observations was 1·40 %. (Difference between duplicates, $(a-b)$, was expressed as a percentage of the mean, $(a+b)/2$. Standard error as percentage is $\sqrt{[\Sigma(a-b)^2/n]}$, where n is the number of pairs.)

Interference by other ions

Aqueous solutions of $(NH_4)_2HPO_4$, KCl, and $CaCl_2$ were prepared to contain 3 mg. of (NH_4), K and Ca/ml., respectively. The (NH_4), K and PO_4 concentrations in these solutions represent the approximate maximum values found in urine; the Ca concentration is much greater. The apparent Na content of each of these solutions was estimated, and the results are summarized in Table 1, column *A*.

Table 1. *Estimation of apparent sodium content of various solutions, separately, and in the presence of standard* NaCl *solution*

(Amount of each cation present at precipitation stage, 0·6 mg. in 4 ml. Values in column *A* for solutions of foreign ions alone (3 mg. each cation/ml.). Values in column *B* for these solutions in the presence of NaCl (3 mg. Na/ml.).)

Solution	*A* Na (mg./100 ml.)	*B* Na (mg./100 ml.)	Apparent increase over Na standard alone (%)
KCl	118	342	14
$CaCl_2$	Nil	300	0
$(NH_4)_2HPO_4$	Nil	300	0
$CaCl_2+(NH_4)_2HPO_4$	Nil	300	0

No precipitate was formed from the $(NH_4)_2HPO_4$ or $CaCl_2$ solutions, but there was a considerable precipitate from the KCl solution. This was not due to Na impurity in the KCl sample used, since an approximate estimation by flame photometry showed a Na content of less than 0·02 g. Na/100 g. KCl.

NaCl (3 mg. Na/ml.) was then added to each solution, and the apparent Na content again estimated. The results in Table 1, column *B*, show that neither the presence of $(NH_4)_2HPO_4$ nor of $CaCl_2$, separately or together, caused any increase in the expected reading of the solution. The presence of KCl, however, caused an increase of 14 %.

To the KCl solution (3 mg. K/ml.) varying quantities of NaCl were added to obtain solutions with a constant K concentration, but with Na/K ratios varying between 9:1 and 1:2. (All Na/K ratios quoted are w/w.) The percentage increase in apparent Na content of these solutions, as compared with NaCl solutions of equal Na concentration (but without KCl), was calculated. In Fig. 1 this percentage increase is plotted against the Na/K ratio for each solution. It will be seen that there is no increase in apparent Na content until the Na/K ratio falls below 5:1.

The apparent Na content of solutions of NaCl plus KCl of constant Na/K ratio (1:1), but of different concentrations, was determined. The percentage increase in reading of the mixed solutions over solutions of NaCl alone of equal Na concentration was estimated. For mixed solutions containing

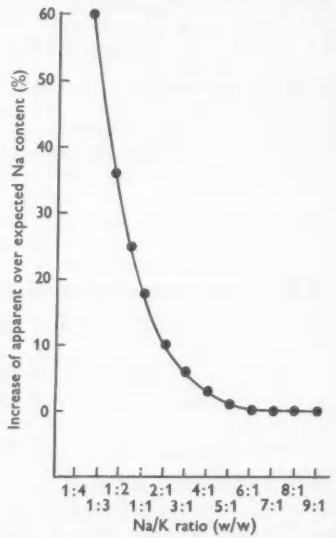

Fig. 1. The effect of varying the Na/K (w/w) ratio by adding increasing quantities of NaCl to a solution of KCl (3 mg. K/ml.). The Na/K ratio is plotted against the percentage increase of apparent over expected Na content.

0·12, 0·30, 0·60 and 0·90 mg. of each cation in 4 ml. at the precipitation stage, the increase in reading was 15, 16, 14 and 17 %, respectively. These two experiments indicate that the apparent increase of Na content is independent of the absolute concentrations of Na and K.

The above findings were applied to urine. Thirteen urine samples were chosen in which analysis by flame photometry indicated that the Na/K ratio lay between 3:1 and 1·5:1. (Flame photometry was used in this and the subsequent experiment as an approximate indication of Na/K ratios only, no high degree of accuracy being assumed.) 3 mg./ml. K (as KCl) was added to each sample to produce Na/K ratios between 1:1·4 and 1:2·7. Sodium was estimated chemically in each sample, both before and after KCl addition. KCl addition increased the apparent Na content of the samples by a mean proportion of 33 %, with a range of 18–47 %.

The above findings suggested that K was being co-precipitated. Accordingly, precipitates were obtained from twelve urine samples in which, by flame photometry, the approximate Na/K ratios were found to cover a range between 1:1 and 1:3. Direct analysis of the washed precipitates was then carried out by flame spectroscopy. Strong emission lines were found in all specimens at 766 mμ., as well as at 589 mμ. Subsequent photography of the emission spectra, using a Hilger medium quartz spectrograph, showed that each line was a doublet, thus confirming them as the well known emission lines of Na (589·0, 589·6 mμ.), and K (766·4, 766·9 mμ.).

Exactly similar results were obtained with the washed precipitate from a solution of NaCl and KCl (3 mg./ml. of each cation), and from the solution itself. Precipitates from solutions of either NaCl or KCl alone showed emission lines at either 589 mμ. or 766 mμ., respectively. Solutions of zinc uranyl acetate showed no emission lines.

The relative intensities of the K emission lines obtained from precipitates of solutions of NaCl and KCl were found, by comparison with the lines obtained from KCl solutions of known concentration, to be approximately consistent with the degree of co-precipitation of K indicated by the chemical estimations.

DISCUSSION

The above studies indicate that, in Dreguss's modification, K interferes with the chemical estimation of Na, both in simple solution, and in urine. This is due to the co-precipitation of K.

High results due to K interference have been reported by Barber & Kolthoff (1928), Butler & Tuthill (1931) and Peters & Van Slyke (1932), but only when more than 25 mg. K was present in the volume taken for analysis. As the K content of urine rarely exceeds 5 mg./ml., this was not considered to be a source of inaccuracy in the urinary estimation. The possibility is, in fact, not mentioned in many subsequent modifications (Salit, 1932; Weinbach, 1935; Bradbury, 1946; King, 1946; Albanese & Lein, 1948).

McCance & Shipp (1931), however, in their modification obtained high results when more than 0·6 mg. K was present in the sample taken for analysis (equivalent to 0·3 mg. in the 7 ml. of solution in which precipitation occurs). Harrison (1951), using this method for tissue fluid, obtained high results due to K co-precipitation, but was able to overcome this by precipitation at between 6° and room temperature, and not at 0°, as recommended by McCance & Shipp.

Using Dreguss's modification for urine, our results indicate that K interference depends on the Na/K ratio, and is independent of the absolute amounts of K present. At low Na/K ratios, K interference occurs even if K is present in smaller amounts than those recommended by McCance & Shipp. Furthermore, K interference is not prevented by precipitation at room temperature as recommended by Harrison, or by the modified precipitation conditions suggested by Van Loon, Likins & Seger (1952).

Methods for overcoming K interference, by removing the ion as $KClO_4$ before Na precipitation, as recommended by Butler & Tuthill (1931) and Dreguss (1939), have not proved satisfactory in our hands. A chance observation led to the finding that the addition of H_2SO_4 prevents the co-precipitation of K from simple solutions of NaCl and KCl. It was hoped that this method could be applied to urine, but consistent results have not been obtained.

As all methods relying on the quantitative precipitation of sodium zinc uranyl acetate are basically similar, it would appear advisable to investigate the possibility of K co-precipitation before using any modification for urine.

SUMMARY

1. A modification of the zinc uranyl acetate method of Dreguss (1939) for the estimation of sodium in urine is subject to interference from potassium.

2. This interference, which is due to co-precipitation of potassium, is dependent on low sodium/potassium ratios, and not on the absolute concentration of potassium.

We would like to record our indebtedness to Dr E. R. Holiday and Dr C. J. O. R. Morris for much help and advice, and to Prof. C. Wilson for laboratory facilities.

REFERENCES

Albanese, A. A. & Lein, M. (1948). *J. Lab. clin. Med.* **33**, 246.
Barber, H. H. & Kolthoff, I. M. (1928). *J. Amer. chem. Soc.* **50**, 1625.
Bradbury, J. T. (1946). *J. Lab. clin. Med.* **31**, 1257.
Butler, A. M. & Tuthill, E. (1931). *J. biol. Chem.* **93**, 171.
Dreguss, M. (1939). *Biochem. Z.* **303**, 69.
Harrison, M. F. (1951). *Biochem. J.* **48**, 283.
Hoffman, W. J. & Osgood, B. (1938). *J. biol. Chem.* **124**, 347.
Holiday, E. R. & Preedy, J. R. K. (1953). *Biochem. J.* **55**, 214.
King, E. J. (1946). *Microanalysis in Medical Biochemistry.* London: Churchill.
McCance, R. A. & Shipp, H. L. (1931). *Biochem. J.* **25**, 449.
Peters, J. P. & Van Slyke, D. D. (1932). *Quantitative Clinical Chemistry*, vol. 2, p. 732. London: Baillière, Tindall and Cox.
Salit, P. W. (1932). *J. biol. Chem.* **96**, 659.
Van Loon, E. J., Likins, M. R. & Seger, A. J. (1952). *J. Lab. clin. Med.* **39**, 148.
Weinbach, A. C. (1935). *J. biol. Chem.* **110**, 95.

The Precision of a Direct-reading Flame Photometer for the determination of Sodium and Potassium in Biological Fluids

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Flame photometry is now well established as a simple method for the determination of sodium and potassium. It has proved particularly valuable for the rapid estimation of these ions in serum, urine and other biological fluids. An early flame photometer was constructed by Barnes, Richardson, Berry & Hood (1945), and since then many instruments have been described (e.g. Hald, 1947; Domingo & Klyne, 1949; Spencer, 1950; Ryssing, 1951). Several commercial models are now available, such as the Beckman DU flame spectrophotometer, the Perkin-Elmer 52A, and in this country the model constructed by Evans Electroselenium Ltd. (Harlow, Essex).

More recently, attention has been directed to the many sources of interference involved in this method. Errors have, for instance, been traced to viscosity (Wynn, Simon, Morris, McDonald & Denton, 1950; Spencer, 1950), and to the presence of other substances, such as urea (Spencer, 1950), glucose (Hald, 1947), acid (Wallace, Holliday, Cushman & Elkington, 1951), and phosphate (Crismon, 1948). Some workers have reported mutual interference between sodium and potassium (Kiers & Speck, 1950; Wallace *et al.* 1951), and others have found difficulty in excluding the calcium emission while measuring sodium (Domingo & Klyne, 1949; Wallace *et al.* 1951).

In view of the increasingly wide use of simple direct-reading flame photometers, especially in clinical laboratories, it was considered appropriate to describe, for comparison, the results obtained with our instrument, which has been in continuous use for 6 years.

Special reference has been made to the nature and limits of interference in simple solutions, in urine, and in serum. In addition, the flame spectrum has been analysed, and the efficiency of the filter systems assessed.

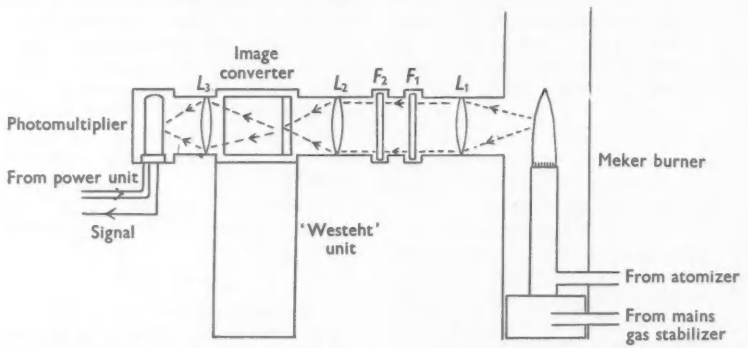

Fig. 1. General layout of flame photometer. L_1, L_2, L_3 are convex lenses. F_1, ON20 filter; F_2, either Na or K filter combinations. Arrows indicate path of light. Distance from flame to photomultiplier, 57 cm.

APPARATUS

The photometer is of the direct-reading type, without internal standard. It consists of the following components: (1) atomizer, (2) burner, (3) optical system of lenses and filters, (4) image converter, (5) photomultiplier and power pack, and (6) galvanometer, with shunt and backing-off circuit. The general layout is shown in Fig. 1.

Atomizer. Oxycillin model, designed for penicillin sprays by Oxygenaire Ltd. (8 Duke Street, London, W. 1), fed by compressed air from a cylinder fitted with pressure and flow gauges. Air flow to the atomizer is maintained at 7 l./min. Trapped spray may be led to waste, or returned to the reservoir, depending on the volume of sample available.

Burner. Meker type Bunsen burner, with flame nozzle of 12 mm. diam., supported in a vertical metal cylinder of bore 5 cm., having opposite the flame a window, 6 × 12 mm., closed by a sheet of mica.

Mains gas is used, the pressure being maintained at 1·5 cm. water by a commercial gas stabilizer Type GLC60D (Evered and Co., 23 Albemarle Street, London, W. 1), which is inserted in the lead to the burner.

Optical system (Fig. 1). This consists of a double condenser with room for insertion of filters (F_1, F_2) in the collimated beam between the two convex lenses (L_1, L_2). A further convex lens (L_3) condenses the light from the image converter on to the photomultiplier. The lenses (3·8 cm. diam. 5 cm. focal length) and filters (5 cm. square) are mounted in a horizontal metal cylinder (4 cm. diam., 55 cm. long) which is supported opposite the window in the burner housing.

Filters. For Na estimations, an interference filter with peak transmission at 590 mμ. (Barr and Stroud Ltd., Anniesland, Glasgow) is combined with an auxiliary filter, Wratten no. 16 (Kodak Ltd., Kingsway, London, W.C. 1). For K estimations, an interference filter with peak transmission at 767 mμ. is used, with a Wratten 88A as auxiliary filter. A heat-resisting filter ON 20 (Chance Bros., St James Square, London, S.W. 1) is used during both estimations.

Image converter (Fig. 1). The photomultiplier (see below) is relatively insensitive to the K 766·0 mμ. radiation. To extend the sensitive range to this region, an image converter is used (no. CV 147 or 148, obtainable from surplus radio stores). This is energized with 2–5 kv (variable) from a Westeht unit (Westinghouse Brake and Signal Co., York Way, London, W. 1). The converter stays permanently in position, but is not energized during Na estimations.

Photomultiplier and power pack. Photomultiplier Type 931A (Radio Corporation of America), energized by 500–1000 v (variable) from a stabilized power unit, Cintel Type 1000/5 (Cinema-Television Ltd., Worsley Bridge Road, London, S.E. 26).

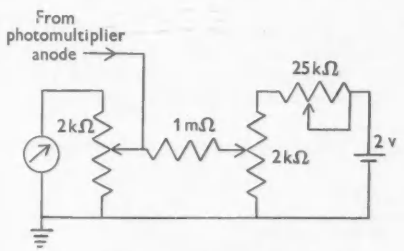

Fig. 2. Shunt and backing-off circuit.

Galvanometer, shunt, and backing-off circuit. Galvanometer, Tinsley Type SS645, 20 cm. scale, sensitivity 1240 mm./μA, slightly overdamped to give a period of about 3 sec. Shunt and backing-off circuits are shown in Fig. 2. The shunt alters the sensitivity of the detection unit, without altering the degree of galvanometer damping. The backing-off circuit is used to balance the small signal from photomultiplier dark current and flame background radiation.

OPERATION

Sodium estimations. The flame is lit, Na filters inserted and the power unit switched on. The instrument is then left for 15 min. Air is turned on, and the flow adjusted to 7 l./min. The gas flow is adjusted so that the cone of the flame is flat. A standard NaCl solution (5 μg. Na/ml.) is atomized, and the galvanometer reading adjusted to nearly full-scale deflexion with the shunt. Distilled water is then atomized, and the reading adjusted to zero with the backing-off circuit. The exact reading of the NaCl standard is taken, and the instrument is then calibrated and ready for use. Unknown solutions diluted to contain 2–5 μg. Na/ml. are atomized, and the readings compared directly with that for the standard (see pp. 216–217).

The usual dilution for urine is 1:500. Dilutions of 1:1000 and 1:200 or less are sometimes necessary. The dilution for serum is 1:1000.

Potassium estimations. K filters are inserted and the image converter switched on. The same routine is followed as with Na estimations, but using a KCl standard (5 μg. K/ml.). For serum, it is usually more convenient to use a more dilute standard (2 μg. K/ml.).

The dilution for urine is usually 1:500, rarely 1:1000. The dilution for serum is 1:100.

RESULTS

Spectroscopic analysis of flame emission

In order to determine whether emissions other than those of Na or K were passing the filter systems, a Hilger D246 spectrometer with glass prism was inserted in the light beam between flame and image converter. The image converter was energized, and the flame spectrum was scanned between 400 and 1500 mμ. The result of a spectral analysis when a solution of KCl and NaCl (containing 5 μg./ml. of each cation) was being atomized is shown in Fig. 3. Similar qualitative results are obtained when serum or urine dilutions are atomized, no additional emission bands being observed. It will be seen from the figure that apart from the Na and K emissions, there are no deflexions at wavelengths shorter than 800 mμ. Between 800 and 1250 mμ., however, there is a broad emission from the flame itself. This remains constant in intensity and distribution whether distilled water, serum or urine dilutions or simple solutions are atomized, or when air alone is blown through.

These results were confirmed over wavelengths shorter than 800 mμ. by photographing the flame emission with a Hilger medium quartz spectrograph, allowing a 20 min. exposure, and using a Kodak IIL plate.

Efficiency of filter combinations

With the monochromator in position, the filters were placed in turn between the flame and the entry slits of the monochromator.

Sodium filters. With the monochromator set to transmit the Na emission at 589 mμ., the Na interference filter was placed in position. The image converter was not energized. The standard NaCl solution (5 μg. Na/ml.) was atomized, and the deflexion adjusted to read 15 cm. A solution of $CaCl_2$ (500 μg. Ca/ml.) was atomized, and the flame

spectrum scanned between 530 and 660 mμ. Apart from a small deflexion centred on 589 mμ. (due to Na impurity in the sample), deflexions were observed at 554 and 624 mμ. only. These amounted to 0·2 cm. each, indicating a relative intensity of Ca lines of less than 0·02 % of Na. Further scanning of the flame emission while a solution of KCl (500 μg. K/ml.) was being atomized showed that the Na interference filter transmitted the weak K emission band at 394 mμ. This was effectively excluded by the Wratten no. 16 auxiliary filter. It was unnecessary to exclude the filter band-passes above 589 mμ., due to the steep fall in sensitivity of the photomultiplier at longer wavelengths.

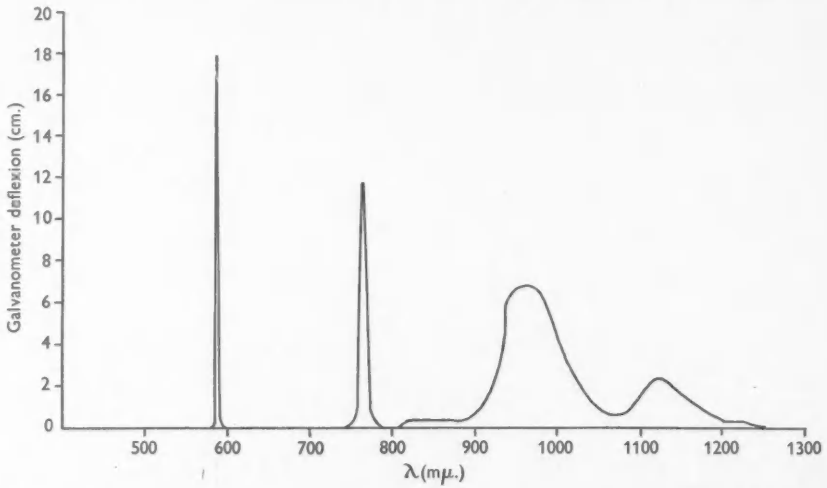

Fig. 3. Tracing of a flame spectral analysis, during the atomizing of a solution containing NaCl or KCl. Na and K emissions at 590 and 766 mμ. respectively. Flame background emission between 800 and 1250 mμ.

Potassium filters. The image converter was energized, and the K interference filter with the Wratten 88A was placed in position. The standard KCl solution (5 μg. K/ml.) was set to read 15 cm. deflexion at 766 mμ., and the flame spectrum scanned as before between 400 and 1300 mμ. No deflexion was observed when NaCl or $CaCl_2$ solutions, containing 500 μg./ml. of the cation respectively, were atomized. In addition, deflexion from the flame background radiation was eliminated.

Interposition of the Chance ON 20 filter alone was found to reduce the flame background radiation by approximately 80 %.

Reproducibility

A number of readings for standard NaCl and KCl solutions, collected over a period of months, are analysed in Table 1. These form a convenient method for determining the linearity of response for standard dilutions, and for estimating the error of dilution and reading under normal working conditions. It was found convenient to express readings for the 2, 3 and 4 μg. K or Na/ml. standards as a percentage of that for the 5 μg./ml. standard.

It will be noted that the mean readings for each standard lie on a curve, a finding which is in agreement with those of other workers (Overman & Davis, 1947; Gilbert, Hawes & Beckman, 1950).

Table 1. *Relation between standard solution readings*

Standard solution (μg./ml.)	No. of readings	Mean reading (as % of that for 5 μg./ml.)	Standard deviation
		Sodium	
2	17	41·15	0·55
3	24	61·82	0·54
4	24	81·33	0·72
5	24	100·00	0·58
			(Mean, **0·60**)
		Potassium	
2	23	40·90	0·71
3	23	62·18	0·65
4	23	81·44	0·57
5	23	100·00	0·78
			(Mean, **0·68**)

However, in the range 1–5 μg./ml. K or Na, the curve is slight, and if the concentration of an unknown solution is calculated by reference to the nearest

standard, the error is negligible. In practice (see p. 215), the reading from the 5 μg./ml. standard only is obtained, the readings for the remaining standards being calculated from Table 1.

Interference in simple solutions

Mutual interference of sodium and potassium. KCl was added to a standard solution of NaCl (4 μg. Na/ml.) in increasing amounts, so that the Na/K ratio (w/w) varied between 4:1 and 1:20. The apparent Na content of these solutions was compared with that of the standard NaCl solution alone. The results are shown in Fig. 4, where the Na/K ratio is plotted against the apparent increase in Na content, expressed as a percentage. There is no increase in the apparent content until the Na/K ratio falls below 2:1. At lower ratios, the apparent content rises to a maximum of 10 % at Na/K ratio 1:7, after which there is no further rise with increasing KCl additions.

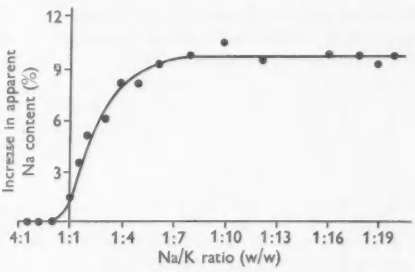

Fig. 4. Effect of increasing KCl additions on the apparent Na content of a standard NaCl solution (4 μg. Na/ml.). The percentage increase in apparent Na content is plotted against the Na/K ratio (w/w).

Similar increases in apparent K content were observed when increasing quantities of NaCl were added to a standard KCl solution under similar conditions.

Interference by other salts found in urine. Solutions of $CaCl_2$, $(NH_4)_2HPO_4$, K_2SO_4 and KCl respectively, were made up as shown in Table 2, the concentrations being the highest likely to occur in urine. The salts were dissolved in 0·1 N-HCl in order to simulate routine urine analysis. (Sufficient acid to obtain the above normality is added to urine samples as a preservative.) These solutions were further diluted 1:500 as for urine, 4 μg. Na/ml. (as NaCl) were added to each, and the apparent Na content compared with that for the 4 μg. Na/ml. standard alone. The results in Table 2 show that each foreign salt produces a significant increase in the apparent Na content. (Allowance was made in each case for a small blank reading due to Na impurity.) The addition of acid in the concentration used here was shown to have no effect on the readings.

Interference by glucose and urea. Glucose and urea were found to have no effect on Na or K readings when present in w/v concentrations up to 0·05 and 0·075 %, respectively. These concentrations are much greater than those likely to be found in urine or serum samples after appropriate dilution.

Interference in urine

Addition experiments. Specimens of urine (51) were chosen with an apparent Na concentration of about 2 μg./ml. after dilution ranging from 1:25 to 1:1000. Sufficient NaCl was added to each to raise the Na concentration by exactly 2·5 μg./ml. The readings before and after NaCl addition were compared. The results in Table 2 show the Na 'found' was equal to Na 'added', within the expected error

Table 2. *Addition of sodium and potassium to various constituents of urine, to urine and to serum*

(1) Substance	(2) Conc. (g./100 ml. 0·1 N-HCl)	(3) No. of observations	(4) Na or K added (μg./ml.)	(5) Na or K found (mean) (μg./ml.)	(6) Mean increase in Na or K (%)	(7) Standard deviation	(8)* Expected standard deviation	(9) Range (%)
				Addition of Na				
$CaCl_2$	0·10	5	2·50	2·70	+8·0	—	—	±0·5
$(NH_4)_2HPO_4$	0·63	5	2·50	2·66	+6·5	—	—	±0·25
K_2SO_4	0·55	5	2·50	2·65	+6·0	—	—	±0·5
KCl	0·40	5	2·50	2·65	+6·0	—	—	±0·5
Mixture of above salts in same concentrations	—	5	2·50	2·75	+10·0	—	—	±1·0
Urine	—	51	2·50	2·50	−0·04	0·05	0·04	—
Serum	—	29	2·50	2·50	±0	0·04	0·04	—
				Addition of K				
Urine	—	28	2·50	2·52	+0·71	0·05	0·05	—
Serum	—	76	2·50	2·61	+4·25	0·05	0·05	—

* Each experiment involves two readings on the galvanometer. The expected S.D. of the result will be $\sqrt{2}.a^2$, where a is the mean standard deviation of the Na or K estimation (Table 1, column 4). The calculated figures appear in column 8.

(column 8). Analysis of the results showed that there was no significant variation referable to the dilution.

These findings are in contrast to Na addition to the composite solution of urinary salts shown in Table 2, column 1, where Na found was 10 % higher than Na added.

A further twenty-eight urine samples were chosen with a K concentration of about 2 μg./ml. after dilution between 1:300 and 1:1000. KCl was added instead of NaCl, as in the previous experiment. Similar recoveries were obtained, as shown in Table 2.

Comparison with chemical methods. Twenty-four estimations of the K content of ten normal urine samples were carried out by the method of Rieben & Van Slyke (1944). Six of these samples were also estimated by a modification of the platinichloride

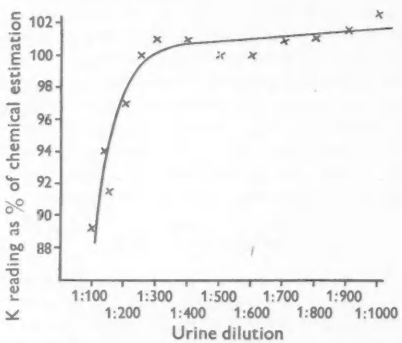

Fig. 5. Effect of dilution on the apparent K content of a urine sample. The increase in apparent K content (expressed as a percentage of the chemical estimations) is plotted against the dilution.

method of Tenery & Anderson (1940), close agreement between the two methods being obtained. These results were then compared with the same number of estimations on the same urine samples by flame photometry, the dilution being 1:500 in each case. Close agreement with the chemical methods was found, the mean variation between chemical and flame analyses being 0·5 % with a range of ± 2·4 %.

Chemical Na analyses were carried out in duplicate on ten futher specimens by the method of Dreguss (1939), as modified by Aitken & Preedy (1953). Urines for these analyses were chosen in which the Na/K ratio (w/w) was 2:1 or above, in order to minimize K precipitation. The absence of significant K precipitation in these particular specimens was established spectrographically (Aitken & Preedy, 1953). The mean variation between chemical and photometric findings was 1 %, with a range of + 1 to − 2·3 %.

Dilution experiments. Eight normal specimens of urine were serially diluted between 1:100 and 1:1000, and the apparent Na and K content of the dilutions was estimated photometrically, by direct comparison with NaCl or KCl standards of concentration 2–20 μg. Na or K/ml. The results were similar in each case. A typical example is shown in Fig. 5 where the apparent K content, expressed as a percentage of the chemical estimation, is plotted against the dilution. Dilution has no significant effect on the expected Na or K concentration over the range 1:250 to 1:1000, the values obtained agreeing with the chemical findings. At lower dilutions, the observed values became progressively lower than expected. Ashing of urine samples at 460–480° for 12 hr. in platinum crucibles before dilution did not alter these findings.

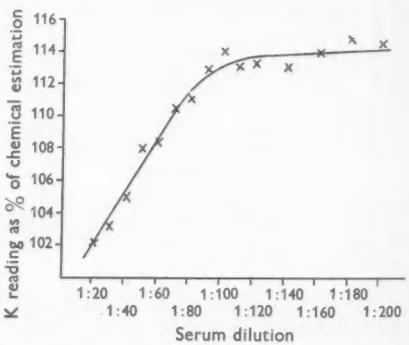

Fig. 6. The effect of dilution on the apparent K content of a typical serum sample. The increase in K reading (expressed as a percentage of the chemical estimation) is plotted against the dilution.

Interference in serum

Addition experiments. Addition of Na (as NaCl) to twenty-nine specimens of serum was carried out as shown in Table 2. Na found was equal to that added within the expected error. The dilution of serum was 1:1000.

However, when similar additions of K (as KCl) were made to seventy-six samples of serum, diluted 1:100, K found was significantly greater than K added, the mean increase being 4·25 %.

Comparison with chemical methods. Using Aitken & Preedy's modification of Dreguss's method, twenty Na results on ten specimens of serum were compared with those obtained photometrically. The mean variation between results by the two methods was 0·7 %, with a range of ± 2·0.

In the case of K, however, when fifty analyses on twenty-five specimens of serum by the method of Rieben and Van Slyke were compared with the photometric results on the same sera, it was found

that the photometric results were higher than the chemical results by a mean of 13%, range ±1·5. Dilutions of 1:100 or 1:150 were used for photometry, the same results being obtained.

Dilution experiments. Serial dilution between 1:700 and 1:1300 of five serum samples caused no significant alteration in expected Na reading.

Dilution was, however, found to have a considerable effect on expected K values. Ten specimens of serum were serially diluted between 1:20 and 1:200. The results were similar in each case, and typical findings with one serum are given in Fig. 6. The apparent K content, expressed as a percentage of the chemical estimation, is plotted against the dilution. The photometric result is progressively depressed at dilutions lower than 1:100, but at this and higher dilutions the reading remains steady at approximately +13% of the chemical figure. Ashing of serum samples (as for urine) did not significantly affect results.

DISCUSSION

With the instrument described, flame photometric analysis of Na and K in simple solutions of NaCl and KCl is reproducible with a high degree of precision which compares favourably with that of most chemical methods (Table 1).

In urine and serum, the validity of flame photometric Na and K analyses has been assessed by addition experiments, by comparison with chemical methods, and by observing the effect of serial dilution.

The great majority of urine samples require dilution between 1:300 and 1:1000. Within this dilution range, it has been shown that recovery of added K or Na is accurate within the expected error limits (Table 2), that photometric results for both cations are in close agreement with those obtained chemically, and that serial dilution has no significant effect on the photometric estimations. It would therefore appear that, within the above range, flame-photometric estimation of Na and K in urine is satisfactory without further correction, and without using the various composite reference standards recommended by several authors (Wynn *et al.* 1950; Mosher *et al.* 1949; Kapuscinski, Moss, Zak & Boyle, 1952).

Some concentrated urines of very low Na content may, however, require dilution below 1:300, and in these, depression of the Na reading might be expected to occur as in Fig. 5. However, eleven such urines, requiring dilution between 1:25 and 1:250, were included in the Na-addition experiments (Table 2). It was found that Na recoveries from these urines were as accurate as for those requiring higher dilution, suggesting that depression of the reading did not, in fact, occur. Unfortunately, this finding could not be confirmed by chemical Na estimations on the same urines, owing to the limitation of the method at low Na/K ratios (Aitken & Preedy, 1953).

Concentrated urines of very low K content, on the other hand, are seldom if ever met with and present no practical difficulty.

With serum Na estimations, Na additions were recovered within the expected limits (Table 2), comparison of flame photometric and chemical results showed good agreement, and there was no apparent interference referable to dilution between 1:700 and 1:1300. Flame photometric estimation of serum Na therefore appears satisfactory.

However, in the case of serum K estimations, the mean recovery of K was 4·25% higher than the K added (Table 2), the mean photometric results were 13% higher than those obtained chemically, and furthermore serial dilutions between 1:20 and 1:200 showed that the photometric analysis is considerably affected by the dilution (Fig. 6).

Serum K estimations are therefore subject to a large systematic error, but although the causes contributing to this finding were not identified, the error is constant to ±1·5%, and determinations to this accuracy can therefore be made by applying an arithmetical correction. It will be noted that the K recovery experiments did not indicate the full extent of the error, possibly due to alteration of the original Na/K ratio by the KCl addition.

For serum K estimations, it would appear advisable to use dilutions between 1:100 and 1:150 where the dilution curve (Fig. 5) is flatter, and the estimations therefore less likely to be affected by alteration in other serum constituents.

The effect of dilution upon photometric determinations of both urine Na and K and serum K is presumably due to altered concentration of inorganic material, since it is not significantly affected by ashing.

Reliable chemical methods for estimating Na and K in urine and serum are laborious and time-consuming, requiring 12–24 hr. for completion. In addition, they are not always free from interference effects (Aitken & Preedy, 1953). Flame photometry, therefore, remains the method of choice where many samples have to be analysed, and where rapid results are required, such as in hospital laboratories. But it appears essential to recognize that the method may be subject to interference, at least with direct-reading instruments. The nature and extent of any interference should, therefore, be established by checking results over a wide range for each type of sample by recovery experiments, by comparison with chemical methods, and by serial dilution.

SUMMARY

1. A direct-reading flame photometer for the estimation of sodium and potassium in urine and serum is described, which has been in continuous use for six years.

2. The flame spectrum has been analysed and the efficiency of the filter combinations assessed.

3. The operation of diluting and reading of samples containing 2–5 μg. sodium or potassium/ml. has a mean standard deviation of 0·03 μg./ml.

4. The validity of sodium and potassium estimations in urine and serum, by direct comparisons with standard solutions of sodium or potassium chloride, has been assessed by addition experiments, comparison with chemical methods and serial dilutions.

5. Urinary sodium and potassium estimations are free from interference at sample dilutions between 1:300 and 1:1000. At lower dilutions readings of both sodium and potassium are depressed.

6. Serum sodium estimations are free from interference, but serum potassium estimations (dilution 1:100) are subject to a systematic error of +13 % of the chemical estimation. Estimations accurate to ±1·5 % can, however, be made by applying an arithmetical correction. At lower dilutions, progressive depression of the potassium reading occurs.

We would like to record our indebtedness to Dr G. H. Beaven for much valuable assistance, to Miss E. H. Aitken for technical help, and to Prof. C. Wilson for laboratory facilities.

REFERENCES

Aitken, E. H. & Preedy, J. R. K. (1953). *Biochem. J.* **55**, 211.
Barnes, R. B., Richardson, D., Berry, J. W. & Hood, R. L. (1945). *Industr. Engng Chem.* (Anal.), **17**, 605.
Crismon, J. M. (1948). *Fed. Proc.* **7**, 24.
Domingo, W. R. & Klyne, W. (1949). *Biochem. J.* **45**, 400.
Dreguss, M. (1939). *Biochem. Z.* **303**, 69.
Gilbert, P. T., Hawes, R. C. & Beckman, A. O. (1950). *Analyt. Chem.* **22**, 772.
Hald, P. M. (1947). *J. biol. Chem.* **167**, 499.
Kapuscinski, V., Moss, N., Zak, B. & Boyle, A. J. (1952). *Amer. J. clin. Path.* **22**, 687.
Kiers, R. J. & Speck, S. J. (1950). *J. Dairy Sci.* **33**, 413.
Mosher, R. E., Boyle, A. J., Bird, E. J., Jacobson, S. D., Batchelor, T. M., Iseri, L. T. & Myers, G. B. (1949). *Amer. J. clin. Path.* **19**, 461.
Overman, R. R. & Davis, A. K. (1947). *J. biol. Chem.* **168**, 641.
Rieben, W. K. & Van Slyke, D. D. (1944). *J. biol. Chem.* **156**, 743.
Ryssing, E. (1951). *Scand. J. clin. Lab. Invest.* **3**, 17.
Spencer, A. G. (1950). *Lancet*, **259**, 623.
Tenery, R. M. & Anderson, C. E. (1940). *J. biol. Chem.* **135**, 659.
Wallace, W. M., Holliday, M., Cushman, M. & Elkington, J. R. (1951). *J. Lab. clin. Med.* **37**, 621.
Wynn, V., Simon, S., Morris, R. J. H., McDonald, I. R. & Denton, D. A. (1950). *Med. J. Aust.* **1**, 821.

The Chemical Nature of the Second Hydrogen Peroxide Compound formed by Cytochrome *c* Peroxidase and Horseradish Peroxidase

2. FORMATION AND DECOMPOSITION

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(*Received* 7 *November* 1952)

Spectrophotometric titration of the second intermediate compound formed by horseradish peroxidase or cytochrome *c* peroxidase and hydrogen peroxide, using the reducing agents ferrocyanide ion, ferrous ion and ferrocytochrome *c* showed that this intermediate compound is one oxidation equivalent above the original ferric state of the enzyme (George, 1952*a*, *b*, 1953), e.g.

$$\text{compound II} + e^- \rightarrow \text{ferric enzyme.} \qquad (a)$$

This compound can therefore be regarded as containing iron with an effective oxidation number of +4, and it differs in two important respects from the classical type of 'enzyme-substrate complex' with which it has long been identified (Chance, 1943, 1949*a–c*, 1951*a*, *b*). First, the substrate, hydrogen peroxide, is not a component part of the structure, because the intermediate is only one oxidation equivalent, and not two, above the ferric state of the enzyme. Secondly, the intermediate cannot dissociate to give the free enzyme and a valency saturated product: it can only be reduced to the free enzyme.

In this paper experiments on the formation and decomposition of the second intermediate compound are described which reveal a further feature

in which it differs from an enzyme-substrate complex. It is not formed from peroxidase and peroxide in an equilibrium system but in a series of irreversible reactions competing for the oxidizing capacity of the peroxide.

METHODS

Symbols. Horseradish peroxidase and cytochrome *c* peroxidase are abbreviated to HRP and CcP.

E_{425} and ΔE_{425} represent the optical density of a solution at 425 mμ. and the optical density increment observed when a reaction takes place. Measurements at other wavelengths are indicated by the appropriate subscript.

Reactions. Reactions were carried out at room temperature, 28–32°, in 1 or 5 cm. cuvettes of a Beckman Model DU spectrophotometer and optical densities measured in the usual way. 1 cm. cuvettes were used unless otherwise stated.

Preparations. Methyl hydroperoxide was prepared according to Baeyer & Villiger (1901) and estimated by iodometric titration. Other experimental details including the preparative methods for the peroxidases, determination of their concentrations, etc., have been fully described in Part 1 (George, 1953). For the complete formation of compound II with HRP and CcP, $\Delta\mu\epsilon_{425}$ has been taken as before to be 0·044 and 0·0405 respectively. The value for CcP includes a correction for the fact that diluent protein in the enzyme preparation gave values for its concentration determined from E_{410} about 8% too high, this figure being estimated from equilibrium measurements on its cyanide complex. Four samples of CcP, referred to as sample 1, sample 2, etc., were used, and four of HRP. One of these had been prepared by Theorell's method (sample 1) and was that employed in part 1: the other three, which were kindly supplied by Dr E. F. Hartree, had been prepared by Keilin & Hartree's more recent procedure (1951). Samples 2 and 3 had been prepared for a few months and sample 4 had been stored for 2 years.

RESULTS

Titration of horseradish peroxidase and cytochrome c peroxidase with hydrogen peroxide

The purpose of these experiments was to determine how the amount of compound II formed depends on the peroxide concentration and to see whether the data support a reaction mechanism in which compound II is in equilibrium with free enzyme and peroxide.

Varying amounts of a 27 μM-H_2O_2 solution were added to an approximately 1·0 μM-HRP solution, sample 1, in 0·01 M-phosphate buffer, pH 5·3, to give peroxide concentrations ranging from 0·2 to 2·0 μM. With the fresh enzyme sample the half-life of compound II was about 5 min., so, to get the amount of compound II formed initially, optical density changes at 390, 395, 400, 420, 425, and 430 mμ. were followed for 12 min. and the curves extrapolated to zero time. The density changes at the three shorter wavelengths gave values for the amount of compound II formed about 14% lower than those obtained at the longer wavelengths. The discrepancy is probably due to small amounts of compound I being present which would have this effect: this is supported by the observation that even with a three- to ten-fold molar excess of peroxide present only 80% of compound II was formed in the absence of added reducing agents like *p*-cresol. For this reason the three higher wavelength values have been taken as the more reliable; the percentages of compound II formed were worked out on the basis that at 425 mμ. an optical density change of 0·044 accompanies the formation of a 1 μM solution. The results are shown in Fig. 1 where the percentage formation is plotted as a function of the H_2O_2/HRP concentration ratio.

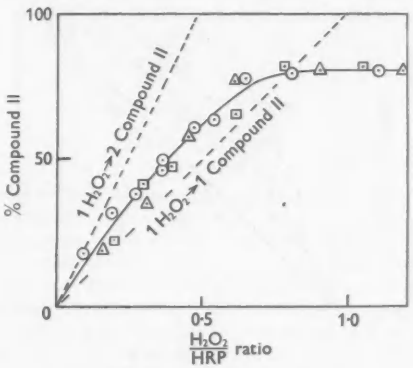

Fig. 1. Titration of HRP with H_2O_2 giving percentage compound II as a function of the H_2O_2/HRP concentration ratio at pH 5·35. ⊡, First titration, 1·0 μM-HRP; △, second titration, 0·20 μM-HRP in 5 cm. cuvette; ⊙, third titration, 1·0 μM-HRP.

The results of a similar titration carried out a few months later, when compound II was found to be much more stable in the system, is also shown together with results of a titration carried out in 5 cm. cuvettes using HRP and H_2O_2 solutions of one-fifth of the previous concentrations, i.e. 0·2 μM-HRP and 0·02–0·25 μM-H_2O_2.

The samples of HRP prepared by Keilin & Hartree's method (1951) gave substantially the same results, except that even less compound II was formed with excess peroxide present. With the fresh samples, 2 and 3, 70 and 60% were formed, respectively. At low values of the H_2O_2/HRP ratio, compound II began to decompose as soon as it was formed and optical densities were plotted against time so that the amount formed initially could be estimated. This was taken to correspond to the extrapolated value 2 sec. from the start of the reaction to make adequate allowance for Chance's (1949*b*) finding that compound II formation can take 0·5 sec. for completion under similar condi-

tions. Similar titration curves to those in Fig. 1 were obtained with both samples, although the extent to which the curve lay above the line corresponding to the formation of 1 mole of compound II from 1 mole of H_2O_2 was less marked. With the old sample, 4, only 50 % compound II was formed with excess H_2O_2 present, and the titration curve lay well below the line. This appeared to be due to less reducing matter being present to bring about the reduction of compound I to II, because the addition of a further amount of peroxide to an enzyme solution in which compound II had been formed and allowed to decompose gave even less compound II than before.

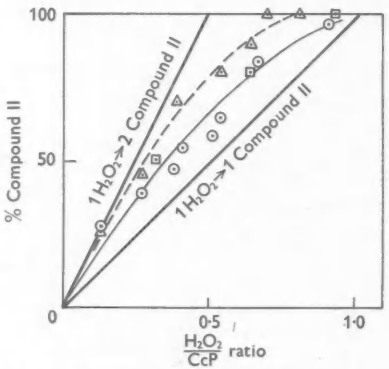

Fig. 2. Titration of CcP sample 2 with H_2O_2 giving percentage compound II as a function of the H_2O_2/CcP concentration ratio at pH 5·35. △, 0·24 μM-CcP; ⊙, 1·20 μM-CcP in 5 cm. cuvette; ⊡, 4·85 μM-CcP.

Using CcP sample 2 a titration carried out in the same manner at three different concentrations of CcP, 4·85, 1·20 and 0·24 μM, gave the results shown in Fig. 2. The percentage compound II formation was obtained from optical density increments measured at 420, 425, 430 and 435 mμ. on the basis that $\Delta\mu\epsilon_{425}$ is 0·0405 for the formation of compound II. The points obtained using 1·2 μM-CcP solution show greater scatter than the others; the reason for this is not known but it was accompanied by similar variations in the maximum optical density changes obtained when excess peroxide was added, such that if the observed change divided by the maximum change is used to calculate the percentage compound II formed the points lie on a much smoother curve.

An attempt was also made to obtain a titration curve at a much higher CcP concentration of 27·5 μM by following the formation of compound II by measuring optical densities at 560 mμ. where compound II has a strong absorption band. But compound II was found to be too unstable under these conditions, with a half-life of about 1 min.

Similar titration results to those given in Fig. 2 were obtained with the three other CcP preparations and, with sample 1, titrations carried out at pH 5·3 and 7·0 were found to give identical results as shown in Fig. 3. With this sample the greatest difference in behaviour was found, a ratio of H_2O_2/CcP of the order of 1·2 to 1·3 being required for the full formation of compound II, as compared to a ratio of 0·7 to 0·95 for the other three samples.

There is clear evidence that under these experimental conditions more than one mole of compound II can be formed for 1 mole H_2O_2, especially at low H_2O_2/peroxidase concentration ratios.

These titration results do not agree with those of previous workers (e.g. Altschul, Abrams & Hogness, 1942) who found the titration curve to lie along and below the line corresponding to the formation of 1 mole compound II from 1 mole H_2O_2. The difference

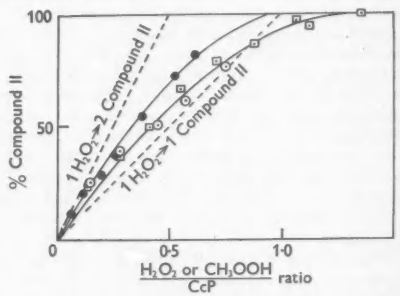

Fig. 3. Titration of CcP sample 1 with H_2O_2 and CH_3OOH giving percentage compound II as a function of the H_2O_2/CcP or CH_3OOH/CcP concentration ratio. 1·0 μM-CcP used. ⊡ and ⊙, H_2O_2 titrations at pH 5·3 and 7·0 respectively; ●, CH_3OOH titration at pH 5·3.

appears to be outside any experimental uncertainties, for instance the precise value for $\Delta\mu\epsilon_{425}$. A possible explanation is that they used a much higher concentration of enzyme that may have contained more reducing matter, thus affecting the formation of compound II, and that slight decomposition of compound II also occurred.

Titration of CcP with CH_3OOH

Within experimental error the spectrum of compound II obtained with CH_3OOH was identical with that for H_2O_2 and the titration curve, shown in Fig. 3, had the same general characteristics indicating that more than 1 mole of compound II can be formed from 1 mole of CH_3OOH.

A test whether compound II is formed in an equilibrium system

In general, a titration curve of the type shown in Figs. 1–3 could arise from one of two mechanisms, either a rapidly established equilibrium where the

Table 1. *The addition of more CcP to a solution of CcP with just enough H_2O_2 present to give 95–100 % compound II*

(7·6 μM-CcP stock solution used in 0·01 M-phosphate buffer, pH 5·35 at 30°. E has been corrected for dilution where necessary to bring all volumes to correspond to a total volume of 2·5 ml. $A5$ is calculated from $A1$. $B4$ is obtained by subtracting $A2$ from $B2$.)

Reference	Particulars of solution, or ΔE	Optical density measurements ($\times 10^2$) at 420 mμ.	425 mμ.	430 mμ.	435 mμ.
$A1$	CcP solution alone, 0·25 ml. in 2·5 ml.	56	43	32	24
2	After addition of 0·05 ml. 50 μM-H_2O_2	79·5	74·5	60	43
3	After addition of 0·10 ml. 7·6 μM-CcP	102	92·5	72	53
4	ΔE resulting from this CcP addition	22·5	18	12	10
5	Calculated ΔE on the basis that the CcP does not react	22·5	17	13	9·5
$B1$	CcP solution alone, 0·35 ml. in 2·5 ml.	78	59·5	43·5	35·5
2	After addition of 0·05 ml. 50 μM-H_2O_2	103·5	96	75·5	55
3	Density resulting from same H_2O_2 addition to previous CcP solution, i.e. $A2$	79·5	74·5	60	43
4	ΔE if H_2O_2 is present initially with CcP	24	21·5	15·5	12
5	Observed ΔE if CcP is added in two portions, i.e. $A4$	22·5	18	12	10
$C1$	Calculated E for 85 % compound II in CcP solution, 0·35 ml. in 2·5 ml.	103·5	95·5	76·5	55·5
2	Observed E in $B2$ above	103·5	96	75·5	55

affinity of the enzyme for the peroxide is not high enough to give 100 % formation at a H_2O_2/peroxidase concentration ratio of 1, or a set of competing irreversible reactions involving at some stage competition for the peroxide or an oxidizing intermediate between the ferric iron atom and other groups such that the full oxidizing capacity of the peroxide does not appear finally in the amount of compound II formed. An experiment to decide between these two possibilities was carried out by adding a further small quantity of concentrated CcP to a solution made from CcP and H_2O_2 where the concentration ratio was chosen to give just 100 % compound II formation. If equilibrium conditions prevail then there should have been either 26 or 63 % free H_2O_2 present according to the titration curve, depending on whether 1 mole of H_2O_2 forms 1 or 2 moles of compound II, and so further addition of CcP should have resulted in the formation of more compound II. If the system is not in equilibrium then there should have been no peroxide left and addition of CcP should have produced no more compound II. Table 1 gives the results of the experiment in which optical density measurements were made at 420, 425, 430 and 435 mμ. A comparison of the optical densities in lines $A4$ and $A5$ show that within experimental error the added CcP did not react to form any additional compound II. A check on the validity of this result is given by a second experiment in which peroxide was added to the combined amounts of CcP: a comparison of lines $B4$ and $B5$ show that more compound II was formed under these conditions.

Finally in lines $C1$ and $C2$ the results of a calculation are given showing that this last result is in accord with the appropriate titration curve, where, with the operative concentration ratio of H_2O_2/CcP 85 % compound II should have been formed. The curve referred to here is one based on the optical density increment for 100 % compound II formation determined for this particular enzyme sample and not on the mean value used elsewhere. This procedure is clearly essential in a calculation of this kind.

The effect of ferrocyanide ion, present initially, on the formation of compound II

It can now be appreciated that compound II resembles the metmyoglobin-peroxide compound in two important chemical aspects. Both compounds are reducible to the ferric haemoprotein in a one-equivalent step, and both are formed from peroxide in non-equilibrium systems resulting in the loss of some of the oxidizing capacity originally present in the proxide if only the actual amount of intermediate compound formed is considered. With metmyoglobin, George & Irvine (1951, 1952) found that ferrocyanide, added initially, reacted with a transient oxidizing species possessing one oxidizing equivalent, which was produced along with the metmyoglobin intermediate compound, and that on this basis the full oxidizing capacity of the peroxide could be accounted for.

Results of similar experiments carried out with CcP are given in Fig. 4, where the optical density of the solution at 425 mμ. is plotted against time for three solutions in which peroxide was added to give 0·80 μM with 0·79 μM-CcP and 0·33, 0·65 and 1·08 μM-ferrocyanide, respectively. The rapid fall in optical density observed shows clearly that only a little

ferrocyanide is used up during the formation of compound II and that the greater part remains to bring about its reduction. The initial concentration of compound II has been obtained by extrapolation as shown in Fig. 4; together with the final concentration this value has been used to calculate the ferrocyanide used in the subsequent reduction of compound II. Subtraction from the concentration of ferrocyanide initially present gives that used up during the formation of compound II, and thus an estimate of the oxidizing capacity of the peroxide which can be accounted for by these two processes. Table 2 shows that with the highest ferrocyanide concentration only 74% of the peroxide is accounted for. Higher concentrations could not be employed because they would have completely reduced all the compound II making the calculation impossible. There is thus a marked quantitative difference between the reactions of metmyoglobin and CcP with hydrogen peroxide which is probably due to the participation of compound I in the latter reaction. This will be discussed later. There can be little doubt, however, that ferrocyanide does compete in the reactions leading to the formation of compound II, for the amount which reacts is approximately proportional to its initial concentration.

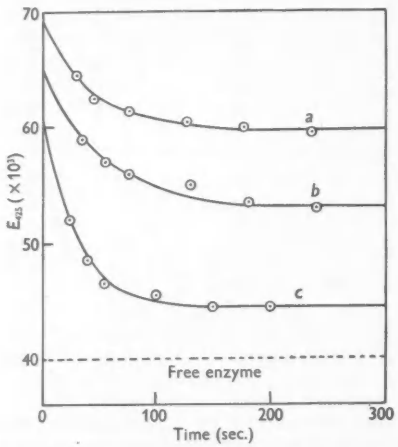

Fig. 4. Optical density changes at 425 mμ. when H_2O_2 to give a 0·80 μM-solution is added to a mixture of 0·79 μM-CcP and (*a*) 0·33 μM-, (*b*) 0·65 μM- and (*c*) 1·08 μM-ferrocyanide, respectively, at pH 5·3.

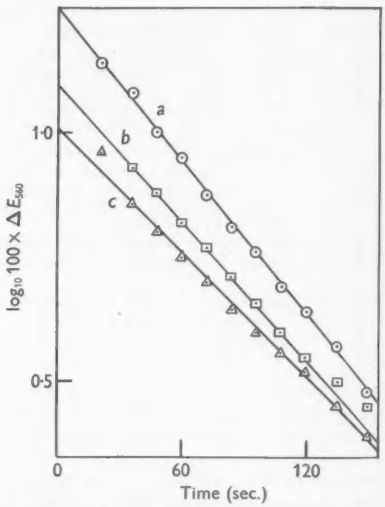

Fig. 5. Plots showing the first-order kinetics of the decomposition of CcP compound II at 30° obtained from optical density measurements at 560 mμ. after several successive amounts of H_2O_2 had been added and allowed to decompose in the solution. The lines *a*, *b* and *c* are for the first three experiments, respectively, listed in Table 3 where full details are given.

The spontaneous decomposition of CcP compound II

As mentioned earlier, the titration of 27·5 μM-CcP with H_2O_2 was found to be impracticable because compound II decomposed rapidly. However, it was possible under these conditions to determine whether this spontaneous decomposition was affected by several additions of peroxide as Chance (1949*b*) observed with HRP. The decomposition was found to be a first-order reaction as shown in Fig. 5, where $\log_{10} \Delta E_{560}$ plotted against time is

Table 2. *The effect of adding* H_2O_2 *to give* 0·80 μM-*solution in a mixture of CcP and ferrocyanide, observed by measuring optical density changes at* 425 mμ.

(All concentrations in μM. The data in columns 2 and 3 are obtained from the curves in Fig. 4.)

Total ferrocyanide concentration	Compound II formed initially	Compound II reacting subsequently with ferrocyanide	Difference between concentrations in columns 1 and 3	Oxidizing equivalents accounted for
0·33	0·67	0·21	0·12	0·79 (49%)
0·65	0·59	0·29	0·36	0·95 (59%)
1·08	0·50	0·39	0·69	1·19 (74%)

Table 3. *First-order decomposition velocity constants at 30° for CcP compound II, after several successive amounts of H_2O_2 had been added and compound II allowed to decompose, obtained from optical density measurements at 560 mμ.*

(Originally there were 0·0686 μmoles CcP present in 2·5 ml. 0·01 M-phosphate buffer, pH 7·0 (i.e. 27·5 μM-CcP); dilution by the peroxide brought the final volume up to 3·02 ml. The kinetic plots for the first three velocity constants are given in Fig. 5 (*a–c*) respectively.)

No. of successive H_2O_2 additions	μmoles H_2O_2 previously added and decomposed	μmoles H_2O_2 at start of kinetic experiment	μmoles H_2O_2 decomposed at end of experiment = p	Ratio: p/μmoles CcP	$k \times 10^2$ sec.$^{-1}$
6	0·156	0·12	0·276	4·0	1·15
8	0·396	0·12	0·516	7·5	1·05
11	0·756	0·12	0·876	12·7	0·96
12	0·876	0·60	1·48	21·5	1·06
14	2·08	0·60	2·68	39	0·95
16	3·68	1·00	4·68	68	0·82

linear, ΔE_{560} being a measure of the compound II remaining at any time. In all, sixteen additions of peroxide were made, and compound II was allowed to decompose almost completely (97 %) before the next addition. Finally, 4·68 μmoles H_2O_2 had been added and decomposed in the solution compared to the 0·0686 μmole CcP present. Table 3 gives a selection of the first-order constants obtained throughout the series of additions showing that there is only a very slight indication that compound II became more stable.

The effect of fluoride and cyanide ions on the stability of compound II

Chance (1952*a*) has observed that when sodium fluoride is added to a solution of HRP and excess H_2O_2 which shows the spectrum of compound II, this spectrum is replaced by that of compound I. In general, two types of explanation could be advanced. First, that the fluoride alters rapidly reversible equilibrium conditions in the system such that a new state of equilibrium is reached favouring a high concentration of compound I rather than compound II. Secondly, that in a non-equilibrium system the transition from compound I into compound II becomes rate limiting in the presence of fluoride.

Inherent in the second explanation is a loss of oxidizing capacity in the system, that is destruction of peroxide accompanying the decomposition of compound II, and either an increase in the decomposition rate of compound II in the presence of fluoride or a decrease in the rate of formation of compound II from compound I. The following experiments carried out with CcP support the second type of explanation.

(1) Preliminary tests with CcP and fluoride at pH 5·3 showed that about 200 μM-sodium fluoride was required to give full conversion of CcP into its fluoride complex, the spectrum of which was given previously (George, 1953). The addition of fluoride to a 0·82 μM-CcP solution, containing 176 μM-H_2O_2 at pH 5·3 to give 7·35 mM-sodium fluoride, resulted in the absorption band at 420 mμ. decreasing in intensity and spreading to the shorter wavelength side characteristic of compound I. This confirms that CcP undergoes the same change that Chance observed with HRP: but reduction with ferrocyanide gave evidence that considerable enzyme destruction also occurs, for a very diffuse band of high intensity was obtained, unlike the sharp band characteristic of the CcP-fluoride complex at 407 mμ. This destruction indicates that the enzyme is going through an oxidation-reduction cycle and that no mere shift of an equilibrium has occurred.

(2) When a concentrated fluoride solution was added to CcP containing just sufficient H_2O_2 to convert all the CcP into compound II with a resulting fluoride concentration of 170 μM, rapid decomposition of compound II occurred, and by 7 min. complete conversion into the fluoride complex had taken place. The addition of a further portion of peroxide equal to that used initially gave momentarily about 50 % of compound II which then decomposed rapidly as before.

(3) Using a lower fluoride concentration the full formation of compound II could be observed even in a solution where its subsequent decomposition was still rapid. This is shown in Fig. 6 where the optical density at 420 mμ., the isobestic point for CcP and its fluoride complex, is plotted against time. To a solution containing 0·76 μM-CcP and 53 μM-sodium fluoride, concentrated peroxide was added at intervals giving 1·2, 0·8 and 1·2 μM-H_2O_2 in the cuvette. The curves in Fig. 6 show that the transitory formation of compound II was 100, 85 and 100 % respectively, in good agreement with the previous titration of the CcP sample which gave 100, 90 and 100 % for these concentrations. At pH 7·0 similar results were obtained except that slight destruction of the CcP was observable by a drop in the final optical density of the solution.

It is clear from these experiments that when the minimal amount of H_2O_2 has been added to convert the CcP into compound II, on the addition of fluoride compound I does not then appear in the system, and that a step in the overall reaction is isolated in which a decomposition of compound II occurs, accelerated in the presence of fluoride, giving the CcP-fluoride complex as the end product. Equally important is the conclusion that the system is not an equilibrium one, since the formation of compound II followed by its decomposition without any

further additions being made to the system (Fig. 6) means that at least one component is being used up. This cannot be the peroxidase because the spectrum of the fluoride complex appropriate to the concentrations used appears at the end of the experiment. Hence peroxide must be destroyed via the decomposition of compound II.

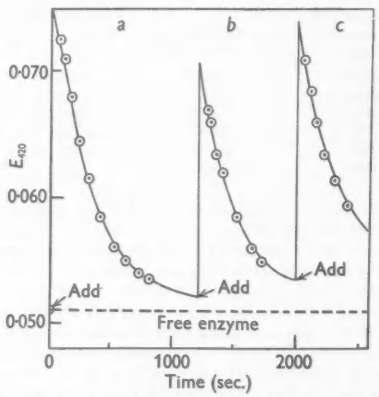

Fig. 6. Optical density changes at 420 mμ., the isobestic point for CcP and its fluoride complex, when H_2O_2 is added to give 1·2, 0·8 and 1·2 μM in a solution containing 0·76 μM-CcP and 53 μM-NaF, the addition being made at times 0, 1200 and 2000 sec., respectively.

The kinetics of this reaction were investigated by measuring optical density changes at 420 mμ. The reaction was found to be first order, as shown in Fig. 7, like the uncatalysed decomposition. At the highest fluoride concentration used, 7·5 mM, the velocity constant was 8·6 times greater than with no fluoride present, that is the half-reaction time was 50 sec. compared with about 7 min. The first-order constants were themselves a linear function of the fluoride concentration (Fig. 7, inset). This suggested that there might be a correlation between the velocity constant k_{fl} for the reduction:

compound II + fluoride (+ reducing agent)
$$\xrightarrow{k_{fl}}$$ CcP-fluoride complex,

and the equilibrium constant for the formation of the fluoride complex

$$\text{CcP} + \text{fluoride} \overset{K_{fl}}{\rightleftharpoons} \text{CcP-fluoride complex.}$$

This was checked by comparing velocity constants and equilibrium constants at pH 5·3 and 7·0. The equilibrium constant is dependent on pH, varying approximately inversely with the hydrogen-ion

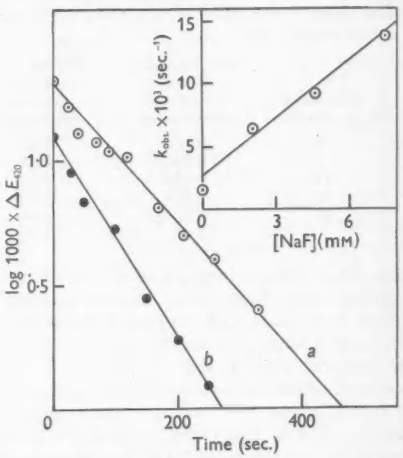

Fig. 7. The first-order decomposition of compound II at pH 7·0 in the presence of (*a*) 2·10 mM- and (*b*) 4·6 mM-NaF and the variation of $k_{obs.}$, the first-order constant so obtained, with the concentration of NaF used.

concentration, and Table 4 shows the velocity constant to have the same type of variation, the ratio K_{fl}/k_{fl} being approximately constant. There thus appears to be a correlation between the affinity of the fluoride for the ferric haemoprotein and the velocity constant in the catalysed reduction of the higher oxidation state to the ferric state. In a similar way cyanide, which also has a high affinity for peroxidase, should also increase the rate of reduction of compound II. This was found to be so, although the experimental error in the determination of the velocity constants was greater because of the smaller optical density increment on going from compound II to the cyanide complex (George, 1953). The equilibrium constant for the formation of the cyanide complex was found to be relatively pH independent and k_{cyn} showed similar behaviour as shown in Table 4.

A further experiment was made using azide as the complexing agent which was found to have, at 30°, reasonably high equilibrium constants of

Table 4. *Correlation between the velocity constants* k_{fl} *and* k_{cyn} *for the catalysed decomposition of CcP compound II in the presence of fluoride or cyanide, and the equilibrium constants* K_{fl} *and* K_{cyn} *for the fluoride and cyanide complex formation with the enzyme itself under the same experimental conditions, at* 30°

pH	K_{fl} (mole^{-1})	k_{fl} (mole^{-1} sec.$^{-1}$)	K_{fl}/k_{fl} (sec.)	K_{cyn} (mole^{-1})	k_{cyn} (mole^{-1} sec.$^{-1}$)	K_{cyn}/k_{cyn} (sec.)
5·3	$1·3 \times 10^5$	32	$4·1 \times 10^3$	$2·45 \times 10^5$	16	$1·5 \times 10^4$
7·0	$4·2 \times 10^3$	2·3	$1·8 \times 10^3$	$4·4 \times 10^5$	10·2	$4·3 \times 10^4$

$1{\cdot}2 \times 10^4$ M^{-1} at pH 5·3 and $1{\cdot}2 \times 10^3$ M^{-1} at pH 7·0, but no satisfactory indication of any increased rate of reduction could be obtained.

DISCUSSION

The oxidation-reduction properties of compound II established in the previous paper confirm the suggestion made recently (George, 1952*a*, *b*) that the transition from compound I to compound II is a reduction process, e.g.

$$\text{compound I} + AH_2 \rightarrow \text{compound II} + AH, \quad (b)$$

where AH_2 represents either reducing groups present in the enzyme preparation or the deliberately added reducing agent. This mechanism explains why the transition is faster with some enzyme preparations than others for the amount and kind of trace reducing matter could vary, and it explains too why the transition is speeded up by all reducing agents that react with compound II (Chance, 1949*b*).

Although an equilibrium mechanism of the type

$$\text{Per. OH} + H_2O_2 \rightleftarrows \text{Per. OOH} + H_2O, \quad (c)$$

for the overall formation of compound II can account for a titration curve deviating from the ideal linear form obtained when a very stable complex is formed, such a mechanism is ruled out because the position of the titration curves in Figs. 1 and 2 is independent of the absolute concentration of the reactants, quite apart from the fact that the mechanism is incompatible with the one equivalent reduction of compound II. The experiment in which a further amount of CcP was added to a mixture of CcP and hydrogen peroxide, such that just 100% of compound II had been formed, showed that no peroxide was left to form more compound II, and therefore a set of irreversible reactions competing for peroxide or other oxidizing intermediates must be responsible for the titration behaviour observed. This conclusion in no way depends on the other feature of the titration curves, namely the apparent production of more than 1 mole of compound II from 1 mole of peroxide, which is also inexplicable in terms of the above equilibrium reaction. This behaviour was found with all the CcP samples and three out of four HRP samples, although the result in the case of HRP depends on the validity of the extrapolation used to determine the initial concentration of compound II. The fact that one HRP sample did not show this behaviour and that it was not previously observed with CcP may be due to different amounts of reducing matter being present in the preparations or reducing matter with a different activity. The varied properties this reducing matter may possess is shown up by the changing stability of compound II in the HRP systems as the preparations are kept, and by the full formation of compound II with CcP in contrast to the 50 to 80% formation observed with HRP.

The titration curves suggest that in the limit 2 moles of compound II can be formed from 1 mole of peroxide, which would be possible in terms of oxidizing equivalents since peroxide possesses two oxidizing equivalents and compound II is one equivalent above the ferric state of the enzyme. For this to occur the net reaction

$$\text{ferriperoxidase} + \text{compound I} \rightarrow 2 \text{ compound II}$$

must also be thermodynamically possible, and the tentative values of the potentials of the two couples compound I/compound II and compound II/ferriperoxidase, which were given in Part 1, are in agreement with this net reaction proceeding with a decrease in free energy corresponding to 0·3–0·6 v. However, it is clear that this net reaction is slow, or can only proceed via the reducing matter present, e.g.

$$\text{compound I} + AH_2 \rightarrow \text{compound II} + AH, \quad (b)$$

$$\text{ferriperoxidase} + AH \rightarrow \text{compound II} + AH_2, \quad (d)$$

otherwise 2 moles of compound II would invariably be formed for each mole of peroxide reacting. The observation that, with HRP, compound II is never formed fully except when reducing agents are deliberately added shows they play an essential role. A mechanism involving (*b*) and (*d*), together with other reactions competing for the AH radical, could account for the type of titration curve obtained. These considerations have an important bearing on the kinetics of peroxidase action. Since the production of compound II is not simply a function of the enzyme and peroxide concentrations but also involves a reducing agent there is no necessary correlation, like that which follows from the Michaelis-Menten enzyme-substrate complex mechanism, between the titration curve obtained in the absence of an added reducing agent and the kinetics of the system when one is present, since the added reducing agent may, in the latter case, be in part or entirely responsible for the reduction of compound I to compound II.

The decomposition of compound II

Since compound II is one oxidizing equivalent above ferriperoxidase there is no possibility of a dissociation like (*c*) above liberating ferriperoxidase, for no stable entity comprising half an oxygen atom or a hydrogen atom exists. This is a further distinction between compound II and the Michaelis-Menten type of enzyme-substrate complex. Compound II can only return to ferriperoxidase by reduction.

The spontaneous decomposition of compound II, although following first-order kinetics as shown in

Fig. 5 above, is presumably pseudo first order, for the magnitude of the velocity constant is dependent on the absolute concentration of compound II, that is compound II is more stable the lower its concentration. This would follow if the reducing matter bringing about its reduction were associated with the enzyme preparation either as trace impurities or as reducing groups on the enzyme protein itself such that the reducing group on one enzyme molecule reacts with the compound II group on another. Compound II formed from the CcP sample did not become more stable after the addition of a few equivalents of peroxide, unlike HRP (Chance, 1949*b*). A possible reason for this is the greater concentration of diluent protein, about 80%, providing a reservoir of reducing groups.

The experiments illustrated in Figs. 6 and 7 and Table 4 show that sodium fluoride brings about an increase in the rate of reduction of compound II giving as the reaction product the CcP-fluoride complex, and that potassium cyanide shows corresponding behaviour. Had cyanide alone shown this effect a likely explanation would have been the oxidation of cyanide ion to cyanogen for the oxidation-reduction potentials are favourable. But the observed hydrogen ion independence of the reaction is against this mechanism for in the pH range 5·3–7·0 an inverse relationship between the rate and the hydrogen-ion concentration should follow, arising from the increase in the cyanide-ion concentration. Oxidation of the undissociated hydrogen cyanide molecule would fit the results, but the similar behaviour found with fluoride calls for a more general mechanism since on energetic grounds the oxidation of either hydrogen fluoride or fluoride ions is ruled out. The correlation shown in Table 4 between the velocity constants k_g and k_{cyn} and the equilibrium constants K_g and K_{cyn} suggests that the velocity constant is larger when the overall free-energy change in the reduction process is larger. For example, the free-energy change in the reaction

$$\text{compound II} + e^- + \text{fluoride} \rightarrow \text{CcP-fluoride complex}$$

is 7·1 kcal. more negative than the straight reduction in (*a*) at pH 5·3 and 30°. A possible mechanism for such an effect is a partial binding of fluoride in the activated complex lowering the free energy of activation. The pH dependencies of the equilibrium constants for the formation of the CcP fluoride and cyanide complexes are similar to those for HRP (Chance, 1952*a*) and favour a combination between CcP and the undissociated acid. The mechanism of this is not known with certainty. It is different from ferricytochrome *c* where the cyanide ion is bound (George & Tsou, 1952) and may be different from methaemoglobin, although there is some divergence of opinion as may be seen in Lemberg & Legge's (1949) review of the earlier work and more recent data where the pH dependence of the dissociation of methaemoglobin-cyanide does not conform to that expected if the cyanide ion were bound to the iron (Chance, 1952*b*). Clearly a detailed mechanism for the decomposition of compound II with fluoride or cyanide present must await the solution of this structural problem.

In a recent paper Chance (1952*a*) reports qualitative observations on the effect of hydrochloric acid decreasing the stability of compound II in solutions of pH 2·0 which may involve a similar mechanism, since hydrochloric acid combines with HRP under these conditions.

A comparison of the peroxide reactions of peroxidase, catalase, and metmyoglobin

The knowledge of the oxidation-reduction properties of the intermediate compounds and the way they are formed from hydrogen peroxide affords an interesting comparison between these three haemoproteins. Keilin & Hartree (1945) showed the catalase-hydrogen peroxide system to be capable of oxidizing simple primary aliphatic alcohols to aldehydes with high catalytic efficiency. Peroxidase is unable to do this and likewise catalase does not bring about the rapid oxidation of phenols, aromatic amines, etc., which the peroxidase system oxidizes efficiently. Keilin & Mann (1937) found that peroxidase compound II reacted rapidly with these reducing agents, and in extensive kinetic studies Chance (1949*d*) showed that compound II is the rate-limiting intermediate in peroxidase reactions whereas only compound I is apparently involved in the rapid oxidation of alcohols by the catalase system. But a little compound II is formed in this reaction and Chance (1950) has also shown that compound II is produced in the catalase-ascorbic acid system. He suggested that it was an unreactive enzyme-substrate complex, which played no essential role in the overall reaction, the production of which merely resulted in less enzyme being available to catalyse the oxidation via the formation and reaction of compound I. More recently, however, George (1952*c*) has shown that catalase compound I can be converted to compound II by a number of reducing agents including phenols like *p*-cresol. Taken in all, these observations suggest an alternative explanation for the specificity of the two enzymes based on a difference of degree rather than kind, namely that in both systems compound II follows compound I but in the case of catalase, compound II preferentially reacts again with the partially oxidized reducing agent before the entities separate, thus simulating an over-all two-equivalent process, e.g.

$$\text{compound I} + AH_2 \rightarrow [\text{compound II}.AH] \rightarrow \text{catalase} + A.$$

The small amount of compound II accumulating in the catalase-alcohol system could then be explained if, by diffusion, some did not undergo the further reaction with AH *in situ*.

Although different in spectroscopic properties from compound II of peroxidase, the metmyoglobin compound shows the same one-equivalent reduction to the ferrihaemoprotein and the same type of pH dependence in its reaction with ferrocyanide (George & Irvine, 1951, 1952). These chemical similarities and the fact that both the metmyoglobin compound and peroxidase compound II form when an equal concentration of peroxide is added to the haemoprotein suggest that these are the analogous compounds of the two haemoproteins and that the correspondence is not between the metmyoglobin compound and peroxidase compound III (which requires a large excess of peroxide for its formation) as proposed by Keilin & Hartree (1951) on the basis of spectroscopic characteristics. With such similar chemical properties it is more likely that the differences in physical properties arise from the residual haemoprotein structure, for instance the iron-protein link may involve a different atom in the two cases.

However, there is a very marked difference in the formation of the metmyoglobin compound compared to compound II of peroxidase, for the analogue of compound I does not appear to exist in the case of metmyoglobin. This is probably the reason for the different behaviour observed when peroxide is added to a mixture of the haemoprotein and ferrocyanide. As shown in Table 2, ferrocyanide does not compete at all effectively in the sequence of reactions leading to compound II. In contrast, George & Irvine (1951, 1952) found that with metmyoglobin one equivalent of ferrocyanide present in the mixture initially allowed the formation of one equivalent of the compound to occur on addition of 1 mole of peroxide, although if the compound were formed first, and then treated with the same amount of ferrocyanide, the compound was completely reduced. They explained this result by the formation of a transient oxidizing entity X along with the metmyoglobin compound

$$\begin{array}{l}\text{metmyoglobin} + H_2O_2 \\ \text{(2 oxid. equiv.)}\end{array} \rightarrow \begin{array}{l}\text{compound} + \quad X \\ \text{(1 oxid. equiv.)} \quad \text{(1 oxid. equiv).}\end{array}$$

which could react with the ferrocyanide if present initially but which disappears by reacting with trace-reducing matter if the formation is allowed to proceed independently. A tentative chemical mechanism was put forward in which X is the OH radical and the intermediate compound has a ferryl ion structure

$$Fe_m^{3+}(H_2O) + H_2O_2 \rightarrow Fe_mO^{2+} + OH + H_3O^+,$$

where Fe_m^{3+} represents the ferriprotoporphyrin iron atom of metmyoglobin. The apparent inability of metmyoglobin to form a compound analogous to compound I is evidence against compound I having the structure of an enzyme-substrate complex for there is no obvious reason why structures of the type $Fe^{3+}(H_2O_2)$ or $Fe^{3+}.O_2H^-$ should not be common to both classes of haemoprotein, in view of their comparable reactions with true complexing reagents like fluoride and cyanide, etc., where the physical properties show great similarities and the only difference lies in the hydrogen-ion dependency of their formation. The possibility that the energetics can favour the stabilization of an effective +5 oxidation state of iron in the haemoprotein enzymes but not in metmyoglobin offers a more profound basis for the differentiation.

The new mechanisms discussed above have a considerable bearing on the role of haemoproteins in biochemical systems under the influence of radiation and in the photosynthetic process. In discussing a hypothetical scheme for the reduction of oxygen to water by the interaction of an iron-containing oxidase, Rabinowitch (1945) made use of a resonance-stabilized intermediate $Fe^{2+}{:}O$ or $Fe^{3+}O^-$ which can be seen to have the redox characteristics now established for the metmyoglobin compound and peroxidase compound II.

SUMMARY

1. Spectrophotometric titrations of horseradish peroxidase and cytochrome *c* peroxidase with hydrogen peroxide suggest that more than 1 mole of compound II can be formed for 1 mole of hydrogen peroxide, the figure approaching two at low values of the hydrogen peroxide/peroxidase concentration ratio. Further experiments established that compound II is not formed in any equilibrium system but in a series of irreversible reactions competing for the oxidizing capacity of the peroxide. Reducing groups present in the enzyme system play an essential role in these reactions.

2. Compound II cannot dissociate into ferriperoxidase since its iron atom has an effective oxidation number of +4: only reduction can regenerate ferriperoxidase. This process, which occurs spontaneously with trace-reducing matter present in the enzyme sample, is more rapid if sodium fluoride or potassium cyanide are added and the fluoride or cyanide complex of ferriperoxidase is the end product. There appears to be some correlation between the velocity constant for this enhanced reduction and the equilibrium constant for the fluoride or cyanide complex formation.

3. If a slight excess of peroxide is added to the fluoride complex, compound II is formed and then disappears with reformation of the fluoride complex.

With a larger excess of peroxide some compound I is also formed. The way in which this reaction occurs excludes an explanation based on shifting the position of an equilibrium in the system; instead, peroxide must be used up in irreversible reactions.

4. The peroxide reactions of metmyoglobin, peroxidase and catalase are discussed in terms of the redox properties of the intermediate compounds and the way they are formed.

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REFERENCES

Altschul, A. M., Abrams, R. & Hogness, T. R. (1942). *J. biol. Chem.* **136**, 777.
Baeyer, A. & Villiger, V. (1901). *Ber. dtsch. chem. Ges.* **34**, 738.
Chance, B. (1943). *J. biol. Chem.* **101**, 553.
Chance, B. (1949*a*). *Science*, **109**, 204.
Chance, B. (1949*b*). *Archiv. Biochem.* **22**, 224.
Chance, B. (1949*c*). *Archiv. Biochem.* **21**, 416.
Chance, B. (1949*d*). *J. biol. Chem.* **179**, 1341.
Chance, B. (1950). *J. biol. Chem.* **182**, 649.
Chance, B. (1951*a*). *Advanc. Enzymol.* **12**, 153.
Chance, B. (1951*b*). *The Enzymes*, ed. by J. B. Sumner and K. Myrbäck. New York: Academic Press Inc.
Chance, B. (1952*a*). *J. biol. Chem.* **194**, 483.
Chance, B. (1952*b*). Unpublished results.
George, P. (1952*a*). *Nature, Lond.*, **169**, 612.
George, P. (1952*b*). *Advanc. Catalys.* **4**, 367.
George, P. (1952*c*). *Biochem. J.* **52**, xix.
George, P. (1953). *Biochem. J.* **54**, 267.
George, P. & Irvine, D. H. (1951). *Nature, Lond.*, **168**, 164.
George, P. & Irvine, D. H. (1952). *Biochem. J.* **52**, 511.
George, P. & Tsou, C. L. (1952). *Biochem. J.* **50**, 440.
Keilin, D. & Hartree, E. F. (1945). *Biochem. J.* **39**, 293.
Keilin, D. & Hartree, E. F. (1951). *Biochem. J.* **49**, 88.
Keilin, D. & Mann, T. (1937). *Proc. Roy. Soc.* B, **122**, 119.
Lemberg, R. & Legge, J. W. (1949). *Hematin Compounds and Bile Pigments*. New York: Interscience.
Rabinowitch, E. I. (1945). *Photosynthesis and Related Processes*, p. 294. New York: Interscience.

The Reaction between Metmyoglobin and Alkyl Hydroperoxides

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In a previous paper, George & Irvine (1952) showed that the reaction between metmyoglobin and hydrogen peroxide was inconsistent with an equilibrium of the enzyme-substrate type, i.e. $E+S \rightleftharpoons ES$. In this reaction the metmyoglobin (MetMb) is oxidized by the peroxide, and the resultant compound is one in which iron is in the effective oxidation state of +4.

Since it was known that alkyl hydroperoxides behaved similarly to hydrogen peroxide in forming a red intermediate compound with methaemoglobin (Keilin & Hartree, 1935), the interactions of these peroxides and MetMb were investigated to see whether the MetMb underwent oxidation of the same type.

In this paper it is shown that spectroscopically the intermediate compounds formed by two alkyl hydroperoxides are the same, and identical with that produced by hydrogen peroxide. The experimental evidence supports a reaction mechanism similar to that suggested for the hydrogen peroxide reaction.

Nomenclature

In the previous papers (George & Irvine, 1951, 1952) the intermediate compound was referred to as a 'complex' partly to preserve continuity with the widespread use of this term for the intermediate compounds formed by peroxides in peroxidase and catalase systems, and partly because such compounds, independent of their chemical structure, may be regarded as co-ordination complexes of iron in the sense that more groups are bound to the iron atom than can be accounted for by the primary valencies of 2 and 3 in its ferrous and ferric states.

In enzyme chemistry however the term complex is usually identified with the enzyme-substrate complex of the Michaelis and Menten theory of enzyme action,

$$E+S \rightleftharpoons ES,$$
$$ES \rightarrow \text{products} + E,$$

or with the modification in which the final reaction is written

$$ES + \text{another reactant} \rightarrow \text{products} + E.$$

The formation and subsequent reactions of the intermediate compound formed from metmyoglobin and peroxides do not correspond to the scheme of chemical

reactions implicit in these generalized equations. The combining proportions are different and the same intermediate is formed by different substrates. For these reasons the purely descriptive term 'intermediate compound' will be used in this and forthcoming papers instead of the term 'complex', and in chemical equations it will be represented by Fe_p^{IV}, following the convention of representing the formula of a metallic compound, in which the oxidation state of the metal is known but not the precise chemical structure of the compound, by its usual symbol combined with the appropriate Roman numeral.

EXPERIMENTAL AND MATERIALS

Methyl and ethyl hydroperoxides were prepared by the action of H_2O_2 on dialkyl sulphates according to the method of Baeyer & Villiger (1901). Following the procedure of Stern (1936) the temperature was kept below 10° during the reaction. The crude products obtained on distillation were redistilled three times and the fraction between 96 and 100° was collected. This was neutralized with disodium hydrogen phosphate and stored at 0°. Tests for H_2O_2 by the titanium method showed that in both preparations there was less than 1%.

Before each experiment the stock solution was standardized iodometrically and was then diluted to the desired concentration.

The metmyoglobin was prepared as described previously and its concentration was determined on the basis of its haematin iron, following the procedure of Keilin & Hartree (1952). In our previous paper the concentration had been determined by using as a standard de Duve's (1948) carboxymyoglobin spectrum. His optical densities corresponded to a concentration of 1 g./l. based on a molecular weight of 16 200. In converting his concentration to molar concentration we however used the value 17 000 for the mol. wt. of MetMb. The molar concentration of our sample was therefore too low by about 4·5% and the molar extinction coefficients recorded were correspondingly too high.

Similar buffer solutions were used as in the previous investigation.

Spectrophotometric measurements were carried out with a Unicam quartz spectrophotometer.

RESULTS

Both methyl and ethyl hydroperoxides were found to give similar results in the following experiments. They form intermediate compounds with MetMb which are spectroscopically identical with that produced by H_2O_2, as shown in Figs. 1 and 2. In contrast, however, to the reaction with H_2O_2, the formation of the intermediate compounds by these hydroperoxides when the pH is greater than 9·0 is unattended by side reactions which lead to the destruction of MetMb. However at pH 10·0 a considerable excess of hydroperoxide, 15 to 20 times the concentration of MetMb, is necessary for complete formation of the intermediate.

If the pH is less than 8·0, the same type of behaviour is observed as in the H_2O_2 reaction, namely the appearance in the spectrum of the reaction product of a pronounced band at 590 mμ. in place of a shoulder and of an additional band at 615 mμ. in the CO-protohaemochromogen. These are shown in Figs. 1 and 3. The addition to the MetMb of a reducing agent, such as iodide ion, before the hydroperoxide, greatly reduces the side reaction, as can be seen in Fig. 3. The same effect is obtained by

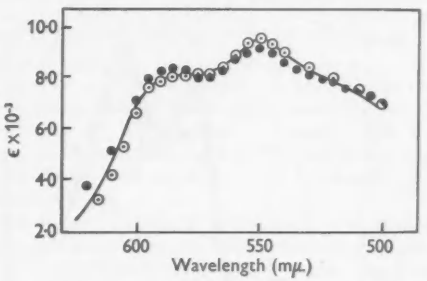

Fig. 1. Visible spectra of the reaction product of MetMb with H_2O_2 and alkyl hydroperoxides, region 650–500 mμ.; smooth curve, using H_2O_2 at pH 8·6; ⊙, using alkyl hydroperoxides at pH 8·6; ●, using alkyl hydroperoxides at pH 6·8.

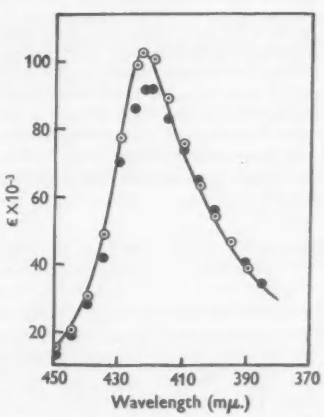

Fig. 2. Soret spectra of the reaction product of MetMb with H_2O_2 and alkyl hydroperoxides; smooth curve, intermediate compound from H_2O_2 and MetMb at pH 8·6; ⊙, alkyl hydroperoxides and MetMb at pH 8·6; ●, alkyl hydroperoxides and MetMb at pH 6·8.

using a large excess of hydroperoxide, e.g. a 25–50 molar ratio of peroxide to MetMb. The reaction product, which is formed very rapidly under these conditions, is identical with that formed at pH 8·6. On standing, however, the haemoprotein is slowly destroyed. This diminution of side reaction was also observed once when an old sample of MetMb was used, when the peroxide present was only just

sufficient to give complete formation of the intermediate compound at these pH values namely, a 3–4 molar excess. This should have represented the optimum condition for the side reaction in view of its suppression by excess peroxide. The reason for this behaviour is unknown, but is possibly due to the production of reducing matter in the stored sample, as will be shown later.

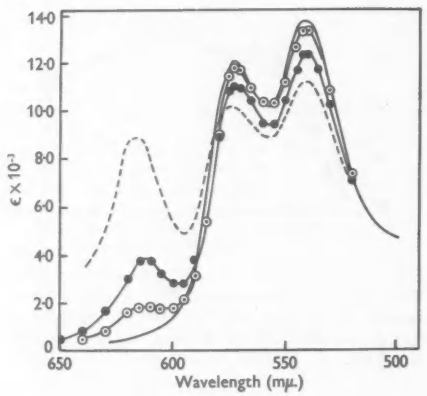

Fig. 3. Spectra of CO-haemochromogen obtained from the reaction product of MetMb and alkyl hydroperoxides under different conditions; smooth curve, from MetMb alone; dotted curve, from product formed at pH 6·8 from methyl hydroperoxide and fresh sample of MetMb; ●, from product formed at pH 6·8 but using an old sample of MetMb; ⊙, from product formed at pH 6·8 from methyl hydroperoxide and fresh sample of MetMb in the presence of iodide ions.

If a calculation is made on the basis of an equilibrium system and that therefore CH_3OOH is available for reaction with the additional MetMb, the intermediate would have been formed in accordance with a CH_3OOH/MetMb ratio of $\frac{1{\cdot}59 \times 10^{-4}}{8{\cdot}7 \times 10^{-5}}$, i.e. 1·84, (from *c* in Table 1). The concentration of intermediate on this basis can be calculated from the percentage formation corresponding to this ratio, as read off from the appropriate graph in Fig. 4 (⊙—⊙), which refers to the

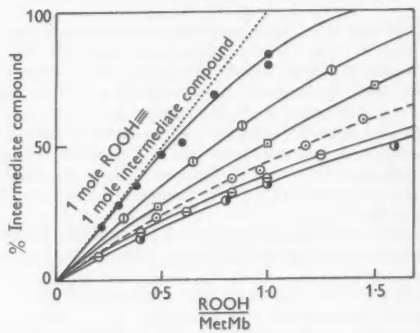

Fig. 4. Percentage formation of the intermediate compound for different ratios of ROOH/MetMb. ●, H_2O_2 and MetMb, $5{\cdot}2 \times 10^{-5}$M, at pH 8·6. ⓘ, CH_3OOH and an old sample of MetMb, $5{\cdot}2 \times 10^{-5}$M, at pH 8·6; □, CH_3OOH and fresh MetMb, $5{\cdot}2 \times 10^{-5}$M, at pH 8·0; ⊖ and ◑, CH_3OOH and fresh MetMb, $5{\cdot}2 \times 10^{-5}$M and $5{\cdot}2 \times 10^{-6}$M, respectively, pH 8·6; ⊙, CH_3OOH and MetMb, from a different preparation, at pH 8·6, MetMb concentration $5{\cdot}2 \times 10^{-5}$M.

Although a 3–4 molar excess of hydroperoxide (ROOH) is necessary for complete compound formation at pH's about 8·0–9·0, and an even larger excess in more alkaline solutions, this does not result from the existence of an equilibrium. This was shown by changing the absolute concentration of MetMb while keeping constant the ROOH/MetMb ratio. The results are shown in Fig. 4. It will be seen that a tenfold change in MetMb concentration hardly affects the percentage compound formation. This would not have been the case if an equilibrium existed.

Further confirmation of the absence of an equilibrium was given by adding to MetMb just sufficient methyl hydroperoxide for full compound formation. When the reaction was complete, a further quantity of MetMb was added. If there were any equilibrium, peroxide should have been available for reaction with this additional quantity of MetMb and this would have resulted in an optical density change corresponding to more compound formation.

same sample with which these experiments were made. The percentage formation is 68%, and this corresponds to an optical density of 0·711, calculated from the measured optical densities of free MetMb and intermediate compound given in (*a*) and (*b*).

This value of 0·711, recorded in (*d*) of Table 1, corresponds, on a concentration basis, to a further formation of $1{\cdot}0 \times 10^{-5}$M intermediate compound or approximately 26% of the added MetMb. The observed optical density after addition of MetMb is 0·670, which is in agreement with the calculated value of 0·671 based on the assumption that the additional MetMb did not react. This experiment proves that even though there was initially a threefold excess of ROOH over MetMb all the peroxide was used up during the formation of the intermediate compound. This points to a reaction mechanism involving irreversible competition for the peroxide and not one involving an equilibrium.

The way in which the percentage formation of the intermediate compound depends on the ratio of

peroxide to metmyoglobin is shown in Fig. 4. Whereas for H_2O_2 a ratio of H_2O_2/MetMb = 1 produces about 80 % of the intermediate, only about 40 to 50 % is obtained for a similar ratio of ROOH/MetMb. This value was reproducible provided fresh samples of MetMb were used. One experiment however, which was carried out on a sample that had been standing at 0° for about 4 months, gave about 60 % compound formation for ROOH/MetMb = 1. Hydrogen-ion concentration was also found to affect the percentage formation. As mentioned before, a considerable excess of alkyl hydroperoxide is necessary for 100 % formation at pH 10·0. In addition, at pH 8·0 the percentage compound formation is greater than that at pH 8·6 for the same ratio of ROOH/MetMb. This is also shown in Fig. 4.

Table 1. *The effect of adding MetMb to a solution of MetMb and* CH_3OOH, *the latter being present at a concentration which was just sufficient to give* 100 % *formation of the intermediate compound, within experimental error*

(pH 8·6; MetMb stock soln. $4{\cdot}15 \times 10^{-4}$ M; CH_3OOH stock soln. $1{\cdot}75 \times 10^{-3}$ M.)

Composition of solution	Optical density at 549 mμ.
(*a*) 1·0 ml. MetMb + 9·0 ml. buffer	0·232 (observed)
(*b*) 1·3 ml. MetMb + 7·7 ml. buffer + 1·0 ml. CH_3OOH. (This gives just 100 % formation of intermediate compound)	0·506 (observed)
(*c*) 1·3 ml. MetMb + 7·7 ml. buffer + 1·0 ml. CH_3OOH: + further addition of 1·0 ml. MetMb	0·670 (observed)
(*d*) As in (*c*) assuming equilibrium and CH_3OOH available for reaction with additional MetMb	0·711 (calculated)
(*e*) As in (*c*) assuming additional MetMb does not react	0·671 (calculated)

Under similar conditions the rate of formation of the intermediate compound from the alkyl hydroperoxides is about 10 times as fast as its rate of formation from H_2O_2. The rate, however, shows the same type of dependence on pH, becoming slower in alkaline solutions. As was found with H_2O_2, the compound on standing reverts to MetMb, but the change is faster than in the H_2O_2 system, about 10–15 % in 30 min. as compared with about 2 % in the same time in the latter system. This regeneration of MetMb is also pH dependent and is faster in acid solutions.

The relative instability of the intermediate in the hydroperoxide systems made quantitative reduction experiments more difficult to carry out than in the H_2O_2 system. In the reduction experiments with ferrocyanide, shown in section (*a*) of Table 2, it was therefore necessary to run a blank and make a correction for the amount of intermediate which disappeared spontaneously. The values of (ferrocyanide ion)/(compound) which are obtained this way are slightly greater than 1·0, but they represent an upper limit for this ratio since this correction for the spontaneous reaction neglects the concurrent ferrocyanide reduction.

It was found that the addition of a little H_2O_2 to the compound after its formation increased its stability, and some reduction experiments were carried out with ferrocyanide under these conditions. Since ferrocyanide reacts only very slowly with

Table 2. *Titration of the intermediate compound formed from MetMb and* CH_3OOH *with ferrocyanide by allowing an excess to react with a limited amount of ferrocyanide at* 18°

(The last three results in section (*a*) are from experiments carried out at pH 6·3, where the intermediate was formed at pH 8·6 and then introduced into strong buffer at pH 6·3. Corrections have been made in column 4 of this section for the spontaneous decomposition of the intermediate. The other experiments in this section and those in section (*b*) were carried out at pH 8·6. No correction for spontaneous decomposition was necessary in section (*b*) as the intermediate compound was stabilized by the addition of a little H_2O_2, the volume of which was small enough to be neglected. All concentrations are in μM.)

	Concentration of ferrocyanide present initially	Concentration of intermediate compound present initially	Measured concentration of intermediate compound left after ferrocyanide reacted	Calculated concentration of intermediate compound reacting with ferrocyanide	Ratio: $Fe(CN)_6^{-4}$: intermediate compound
	(1)	(2)	(3)	(4)	(5)
(*a*)	20·0	40·0	12·0	19·0	1·05
	30·0	38·0	6·0	23·0	1·30
	10·0	32·0	19·0	8·0	1·25
	30·0	32·0	2·0	25·0	1·20
	20·0	27·0	5·0	14·0	1·43
(*b*)	15·0	42·0	28·0	14·0	1·07
	22·5	36·0	15·0	21·0	1·07
	30·0	31·0	1·0	30·0	1·00
	24·0	35·0	10·0	25·0	0·96

H_2O_2, the amount used up is a direct measure of the oxidizing equivalent of the intermediate compound. The values of (ferrocyanide ion)/(compound) which are obtained from these experiments are given in section (*b*) of Table 2. In this table, column 1 gives the molar concentration of added ferrocyanide before reaction with the intermediate compound, the necessary correction having been made for dilution effects, e.g. 1 ml. of ferrocyanide added to 9·0 ml. of intermediate. Columns 2 and 3 are the molar concentrations of the intermediate before and after reaction with the added ferrocyanide, the effect of dilution by ferrocyanide having been taken into account in the case of column 2. Column 4 is the calculated concentration of intermediate which reacts with the ferrocyanide. This is obtained by subtracting from the difference between columns 2 and 3 a correction for the spontaneous decomposition of the intermediate over the same reaction time, as determined from control experiments.

The results in Table 2 show that the intermediate compound has one oxidizing equivalent relative to its reduction to MetMb. It may therefore be considered as a compound in which the iron has an effective oxidation number of +4. Hence the reaction of MetMb with the alkyl hydroperoxides is analogous to the H_2O_2 reaction. In this case it was shown, in addition, that a transient oxidizing entity was produced during the formation of the intermediate according to the equation

$$\underset{\substack{\text{2 oxidizing}\\\text{equivalents}}}{\text{MetMb} + H_2O_2} \rightarrow \underset{\substack{\text{1 oxidizing}\\\text{equivalent}}}{Fe_p^{IV}} + \underset{\substack{\text{1 oxidizing}\\\text{equivalent}}}{X.} \quad (1)$$

Similar experiments were carried out to establish whether a transient oxidizing entity was also formed in alkyl hydroperoxide systems. When a little luminol was added to the MetMb, a bright flash was observed on addition of the hydroperoxide, and this was followed by a dull luminescence. The addition of luminol after the hydroperoxide gave only a dull luminescence. The addition of ferrocyanide before hydroperoxide produced the same type of behaviour as in the H_2O_2 reaction, although there was a quantitative difference. Thus when 1 mol.prop. of ferrocyanide was added to MetMb and 1 mol.prop. of H_2O_2 introduced, no reduction of the intermediate was observed. In the case of the hydroperoxides, however, reduction was observed whenever more than 0·5 mol.prop. of ferrocyanide was present for 1 mol.prop. each of MetMb and ROOH. With less than 0·5 mol.prop. ferrocyanide no reduction occurred and the amount of intermediate produced was the same as that produced in the absence of ferrocyanide.

DISCUSSION

The above results show that the reactions between the alkyl hydroperoxides or H_2O_2 and MetMb are essentially the same. The identical nature of the spectrum of the intermediate compound in both systems, as well as the fact that in each case the intermediate is one oxidizing equivalent above MetMb, suggests that the same intermediate is produced. In both reactions there is no evidence of any equilibrium, the intermediate being produced in an irreversible reaction given by equation (1).

In the presence of added reducing agent the side reaction, which occurs when the peroxides react with MetMb at pH's less than 8·0, is greatly reduced. This reduction of the side reaction is also brought about by excess peroxide. Two conclusions may be drawn from these experiments, first that the peroxide molecule itself cannot be responsible for these side reactions, and, secondly, that the entity responsible is capable of reacting both with reducing agents and peroxide.

In the previous paper it was shown that the intermediate compound does not react with MetMb. The side reaction which occurs in acid solution may therefore be attributed to the transient oxidizing entity which is produced during the formation of the intermediate compound, and the slight differences which are revealed between the reactions of H_2O_2 and the alkyl hydroperoxides to competitive reactions involving this entity.

A reaction scheme which fits the observations and accounts for the differences between the reactions of H_2O_2 and the alkyl hydroperoxides is given by the following equations

$$\underset{\substack{\text{2 oxidizing}\\\text{equivalents}}}{Fe_p^{III} + ROOH} \rightarrow \underset{\substack{\text{1 oxidizing}\\\text{equivalent}}}{Fe_p^{IV}} + \underset{\substack{\text{1 oxidizing}\\\text{equivalent}}}{X,} \quad (2)$$

$$X + ROOH \rightarrow \text{chain reaction leading to destruction of ROOH,} \quad (3)$$

$$X + AH_2 \rightarrow HX + AH\cdot, \quad (4)$$

$$X + \text{MetMb} \rightarrow \text{side reaction products,} \quad (5)$$

where Fe_p^{III} and Fe_p^{IV} represent myoglobin in which the iron is in the effective oxidation states of +3 and +4 respectively; AH_2 represents a reducing agent, which may either be reducing groups on the haemoprotein or reducing matter associated with the preparation; AH· is the half-oxidized form of AH_2; X is a transient oxidizing entity which may be the OH radical or OR radical.

On the basis of these equations, the formation of 1 mole of the intermediate from 1 mole of H_2O_2 at low H_2O_2/MetMb ratios can be explained if reaction (3) is unimportant with respect to (2) and (4).

In the case of the alkyl hydroperoxides, at low ROOH/MetMb ratios less of the intermediate is formed than would be expected if only reaction (1) occurred, and in fact a three to four molar ratio of the peroxide to metmyoglobin is required for its full formation.

This behaviour, which is illustrated in Fig. 4, would follow from the above equations if reaction (3), leading to peroxide destruction, occurs much more readily with ROOH than H_2O_2 and so plays a significant role in the alkyl hydroperoxide system. Since the rate of reaction (3) will depend on the peroxide concentration, it also follows that this reaction will be relatively more important with high as opposed to low peroxide concentrations. Consequently, in a series of experiments in which the peroxide concentration is varied at constant metmyoglobin concentration, much less of the intermediate compound would be expected at high ratios of peroxide to metmyoglobin than would be the case if only reaction (1) occurred.

If the percentage compound formation is then plotted as ordinate against peroxide/MetMb ratio this would result in the bending of the curve towards the abscissa. Fig. 4 shows that this feature is present in both H_2O_2 and ROOH systems, although it is more marked in the latter in agreement with the above indication that in this system reaction (3) occurs more readily.

As mentioned before the side reaction, which occurs if the pH is less than 8·0 in both the H_2O_2 and ROOH reactions, may be attributed to the attack of X on the haemoprotein. This could occur when reaction (5) in the above scheme becomes important relative to (3) and (4). By adding a reducing agent before the peroxide, or by increasing the peroxide concentration, the importance of reactions (4) and (3) may respectively be increased, resulting in the observed diminution of the side reaction.

Above pH 9·0 the ROOH reaction differs from the H_2O_2 reaction in that no side reaction occurs which involves haemoprotein destruction. The fact that in the H_2O_2 system the side reactions in acid and alkaline solutions are different suggests that it is possibly the attack of H_2O_2 itself on the haemoprotein which is responsible for the latter. Whatever the mechanism operating, the absence in the ROOH system in alkaline solution of a side reaction similar to that observed in the H_2O_2 system may be explained by the importance of reaction (3). The destruction of ROOH as a consequence of this reaction would also explain why such a large excess of ROOH, between 15 and 20 times the metmyoglobin concentration, is required to give full formation of the intermediate compound in alkaline solution.

In conclusion, reaction (4) can account for the rapid disappearance of reducing agents when these are added before peroxide, and the scheme as a whole is consistent with the difference observed in these experiments between the H_2O_2 and ROOH systems. Thus, at pH 8·6, a mol.prop. of ferrocyanide as high as 1 was almost entirely consumed whilst the intermediate compound was being formed in a mixture of 1 mol.prop. metmyoglobin and 1 mol.prop. H_2O_2 to which the ferrocyanide had been added before the peroxide (George & Irvine, 1952), whereas, in the experiments with ROOH reported above, if the mol.prop. of ferricyanide exceeded 0·5 not all of it was consumed in this way, but some remained in the solution as shown by the subsequent reduction of the intermediate compound. In the H_2O_2 system, reactions (2) and (4) and not (3) appear to be the important steps under these conditions, whereas with the alkyl hydroperoxides reaction (3) is also important, so that the amount of added reducing agent which is removed would therefore depend in this case on the relative importance of reaction (3) with respect to reaction (4).

The observations on the aged sample of MetMb can be explained in a similar way. For, if reducing matter was formed in the MetMb on standing, the increased importance of reaction (4) by preventing some of the peroxide destruction in reaction (3) would result in an increase in the formation of the intermediate, and, by removing a greater proportion of X, would result in a diminution of the side reaction which occurs in acid solution.

SUMMARY

1. The intermediate compounds which result from the reaction of alkyl hydroperoxides with metmyoglobin are spectroscopically the same, and identical with that produced by hydrogen peroxide.

2. The compound is not formed in an equilibrium reaction but irreversibly according to the equation

$$\text{MetMb} + \text{ROOH} \rightarrow \text{compound} + \text{X},$$

where X is a transient oxidizing entity.

3. At pH's less than 8·0 a side reaction is produced similar to that in the hydrogen peroxide system. Above pH 9·0 no side reaction, which destroys the haemoprotein, is observed; however a large excess of hydroperoxide is required for complete formation of the intermediate.

4. Addition of a reducing agent to metmyoglobin before hydroperoxide reduces the side reaction which otherwise occurs at pH's less than 8·0. The same effect is brought about by using a large excess of peroxide.

5. Reduction with ferrocyanide shows the intermediate to have one oxidizing equivalent relative to metmyoglobin, and the intermediate may thus be considered as a compound in which iron has an effective oxidation number of +4. Its formation and reactions may thus be written in stoicheio-

metric equations using the symbol Fe_p^{IV}, which indicates its general oxidation-reduction behaviour and not a particular chemical structure.

6. A mechanism is proposed for the overall reaction of hydrogen peroxide and hydroperoxide with metmyoglobin involving competition reactions of the transient oxidizing entity, which is produced during the formation of the intermediate compound.

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REFERENCES

Baeyer, A. & Villiger, V. (1901). *Ber. dtsch. chem. Ges.* **34**, 738.
Duve, C. de (1948). *Acta chem. scand.* **2**, 264.
George, P. & Irvine, D. H. (1951). *Nature, Lond.*, **168**, 164.
George, P. & Irvine, D. H. (1952). *Biochem. J.* **52**, 511.
Keilin, D. & Hartree, E. F. (1935). *Proc. Roy. Soc.* B, **117**, 1.
Keilin, D. & Hartree, E. F. (1952). *Biochem. J.* **49**, 88.
Stern, K. G. (1936). *J. biol. Chem.* **114**, 473.

A Spectrophotometric Study of Ionizations in Methaemoglobin

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Whereas Gamgee (1868) was the first to observe a reversible change between two coloured forms of methaemoglobin, it was Hartridge (1920) and Haurowitz (1924) who showed that an acid-base indicator relationship was involved in this equilibrium. Austin & Drabkin (1935) used canine-blood methaemoglobin (MetHb) and a spectrophotometric method for the measurement of its equilibrium, or ionization, constant. They concluded that the ionization was single, that is it involved one H^+ per Fe atom, and that it had a pK′ value of 8·12 at ionic strength $I = 0{\cdot}10$ (and an unspecified temperature).* They also concluded, although this is not shown directly in their data, that at ionic strengths below 0·15 the variation of pK′ with $\sqrt{I}$ is linear with a slope of about $-0{\cdot}6$. Coryell, Stitt & Pauling (1937) titrated bovine MetHb magnetometrically at higher ionic strengths and stated that the linear slope of pK′ with $\sqrt{I}$ is about $+0{\cdot}6$. In this paper it is shown that these results are not contradictory, for the addition of neutral salts favours the ionization of acid MetHb up to $I \simeq 0{\cdot}1$, but beyond this the effect is reversed. A similar behaviour has been described in a previous paper (George & Hanania, 1952, subsequently referred to as Paper 1) which dealt with the ionization of acidic metmyoglobin (acid MetMb) and the evaluation of its approximate heat and entropy of ionization.

No value for the corresponding heat of ionization of acid MetHb is reported in the literature, though Wyman & Ingalls (1941) have taken it to be 13·0 kcal./mole on the assumption that its ionization would be very similar to that of water. This assumption is not borne out by the value $\Delta H = 3{\cdot}91 \pm 0{\cdot}49$ kcal./mole obtained in the present investigation.

* Following the usual convention the symbol pK′ is used here to refer to an experimentally determined equilibrium constant at a finite ionic strength, whereas pK denotes a value obtained by extrapolation to zero ionic strength.

MATERIALS AND METHODS

Preparation and storage of MetHb. Samples of crystalline oxyhaemoglobin (HbO_2) were prepared from the blood of horse, ewe and ram, following the method of Keilin & Hartree (1935). MetHb was prepared by the oxidation of HbO_2 with an excess of $K_3Fe(CN)_6$ not exceeding two equivalents, and was freed from inorganic salts by dialysis. Next, it was salted out by adding $(NH_4)_2SO_4$ to about 65% saturation and was redissolved to make the required stock solution. It was found best to keep haemoglobin as crystallized HbO_2 at about 0°, but MetHb could also be successfully stored for long periods in a 2% salt solution (w/v) kept frozen at −10°. Freeze-dried MetHb appeared to keep indefinitely, but its solutions showed some loss of resistance to alkali denaturation (cf. Keilin & Hartree, 1952). Samples of MetHb prepared simply from salted-out HbO_2 did not keep so well and slight opalescence was observed in dilute solutions at the lowest ionic strengths when the pH was around the isoelectric point.

Except where otherwise indicated, all results quoted in this paper refer to horse-blood MetHb prepared from HbO_2 which had been crystallized once from 20% (v/v) ethanol. MetHb was invariably stored in 2% (w/v) NaCl or 2% (w/v) $(NH_4)_2SO_4$ at low temperatures. Concentrations of MetHb solutions are expressed in terms of their haematin content (1 M≡17 000 g./l.). This was measured as the pyridine haemochromogen according to the method of Keilin & Hartree (1951). The method gave concentrations which accorded well with those based on the standard absorption curve for human carboxyhaemoglobin (de Duve, 1948), discrepancies not exceeding 1·5%.

It should be mentioned that in paper 1, metmyoglobin concentrations were based on the latter standard but a molecular weight of 17 000 was assigned to MetMb instead of the 16 200 used by de Duve. Concentrations determined in

this way were therefore too low by about 4%, and the extinction coefficients were correspondingly too high.

Buffer solutions. The main pH region, 8·0–10·2, was covered by borate buffers made from H_3BO_3-NaOH mixtures. In the pH range 5·8–8·0, phosphate buffers were made from the appropriate NaH_2PO_4, $2H_2O$-NaOH mixtures, and for lower pH values phthalate buffers were used. All solutions were prepared using A.R. chemicals and water which had passed through a column of exchange resin (Bio-Deminrolit FF, Permutit Co. Ltd.), and made up to the same total ionic strength, usually 0·05, with NaCl. It will be shown below that the contribution to I from solutions of MetHb about 5×10^{-5} M is considerably less than 0·001.

Spectrophotometric measurements. Optical densities were measured with a Unicam quartz spectrophotometer, temperature being controlled to $\pm 0{\cdot}2°$ by means of a circulating water device. In the usual type of experiment the total MetHb concentration was about 5×10^{-5} M, and optical densities were measured at 578 mμ. using 1 cm. glass cells. Fig. 2 shows that at this wavelength alk. MetHb has a sharp band with an extinction coefficient $\epsilon = 8610 \pm 80$ while acid MetHb has $\epsilon = 3180 \pm 30$. As a check, the MetHb concentration was varied in a series of experiments by a factor of 200. At the lowest concentrations, $2{\cdot}5 \times 10^{-6}$ M, measurements were made in the near ultraviolet region, at the sharp Soret band, 405 mμ., of acid MetHb. Solutions of the order $5{\cdot}0 \times 10^{-4}$ M were measured at 578 mμ. using 1 mm. optical paths. Some experiments were also made using the 501 mμ. band of acid MetHb.

Measurement of pH was done as described in Paper 1. The pH standard of 0·05 M-borax was taken as 9·21 at 20°, 9·15 at 28° and 9·09 at 37°, and of 0·05 M-potassium hydrogen phthalate as 4·01 at the three temperatures. No further corrections were applied for the change in liquid junction potentials with the changing ionic strength of the solutions.

RESULTS

Spectrophotometric characteristics. The absorption spectra of the acidic and alkaline forms of MetHb have been recorded by several workers, but the extinction coefficients reported are not always in satisfactory agreement. Reference may be made to Horecker (1943) for the infrared and visible, to Austin & Drabkin (1935) for the visible, and to Hicks & Holden (1929) for the ultraviolet region of the spectrum.

A more thorough study of these spectra reveals a subtle dependence, hitherto unobserved, of the spectrum of acid MetHb on ionic strength. Two minor changes in the absorption spectrum of the brown acid MetHb are in fact detectable at sufficiently low ionic strength, and they can be attributed to the following ionizations:

$$H_2Pr.Fe^+(H_2O) = HPr^-.Fe^+(H_2O) + H^+ \qquad pK_1' \simeq 5{\cdot}1, \quad (1)$$

$$HPr^-.Fe^+(H_2O) = Pr^{2-}.Fe^+(H_2O) + H^+ \qquad pK_2' \simeq 6{\cdot}4, \quad (2)$$

which precede the main ionization

$$Pr^{2-}.Fe^+(H_2O) = Pr^{2-}.FeOH + H^+ \quad pK_3' \simeq 8{\cdot}4. \quad (3)$$

This last equilibrium involves the iron-bound water molecule and is responsible for the colour change from brown to red (Keilin & Hartree, 1949; Haurowitz, 1949). The symbols used above were chosen arbitrarily and the charges indicated are purely formal. Pr stands for the globin moiety, $H_2Pr.Fe^+(H_2O)$ and $HPr^-.Fe^+(H_2O)$ being respectively, the conjugate acid and base associated with pK_1'. pK_2' and pK_3' are defined in a similar manner. The location of the ionizable H^+ in Eqns. 1 and 2 is unknown though, presumably, they must be near the iron atom. The haematin-linked property of these two ionizations is operative, spectrophotometrically, at low ionic strength only. At all higher values of I the spectrum of acid MetHb corresponds to that of the species $HPr^-.Fe^+(H_2O)$ alone.

This complicated behaviour is illustrated in Figs. 1–3. Fig. 1 gives some absorption spectra, in the visible range 650–470 mμ., for acid MetHb at pH values below 5·8 and $I = 0{\cdot}02$. A small but definite and reversible change can be seen, in accordance with Eqn. 1. Unfortunately, the onset of irreversible denaturation at pH values below about 4·5 prevents the attainment of a clear spectral definition of the species $H_2Pr.Fe^+(H_2O)$.

Fig. 2 covers the same wavelength region at higher pH values. These spectra are given at $I = 0{\cdot}02$. The one at pH 5·6 represents the species $HPr^-.Fe^+(H_2O)$ and is obtained by extrapolation using the pK′ values which are given below. The absorption curve at pH 6·6 corresponds to about 80% formation of the intermediate species $Pr^{2-}.Fe^+(H_2O)$. The curve labelled $Pr^{2-}.FeOH$ represents the red alk. MetHb at pH 10·2.

The effect of neutral salts is to mask the two ionizations with the constants K_1' and K_2' so that at high salt concentrations, independently of the nature of the salt, the species $Pr^{2-}.Fe^+(H_2O)$ can no longer be detected, and the change identified with the appearance of $H_2Pr.Fe^+(H_2O)$ is much smaller. These two effects are of opposite sense, for it is the spectrum of the conjugate acid of pK_1', and that of the conjugate base of pK_2', which are suppressed. That is, only one species of acid MetHb, that corresponding to $HPr^-.Fe^+(H_2O)$, is operative in the pH range 4·6–7·0 at sufficiently high ionic strength. This point is illustrated in Fig. 3 which gives a composite spectrophotometric titration curve of MetHb at 578 mμ. and where the differentiation between the various ionized forms can be seen clearly.

Determination of pK_2', pK_3' *and* pK_1'. It is convenient to measure an equilibrium constant K_3' which refers to the ionization in Eqn. 3 which may be defined by the equation

$$pK_3' = pH + \log \frac{(\text{acid MetHb})}{(\text{alk. MetHb})}, \quad (4)$$

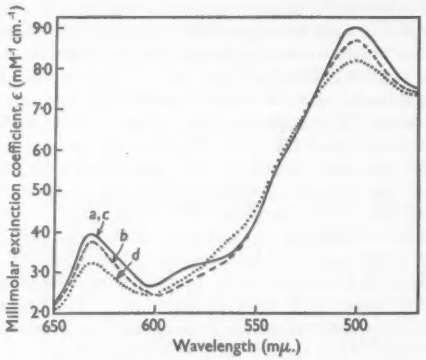

Fig. 1. Absorption spectra of acidic methaemoglobin in the range 650–470 mμ. The solutions are in phthalate buffers at 20° and $I = 0{\cdot}02$. (*a*) pH = 5·72 (continuous line). (*b*) pH = 4·70 (broken line). No change is observed after 1 hr. The spectrum corresponds roughly to the species $H_2Pr.Fe^+(H_2O)$ of Eqn. 1. (*c*) Solution (*b*) diluted with buffer of pH 6·2 so that the pH of the resulting solution is 5·68: when corrected for dilution the spectrum coincides, within experimental error, with that of (*a*). (*d*) pH 4·39, the spectrum was taken within 20 min. and a slow irreversible change in the absorption was observed.

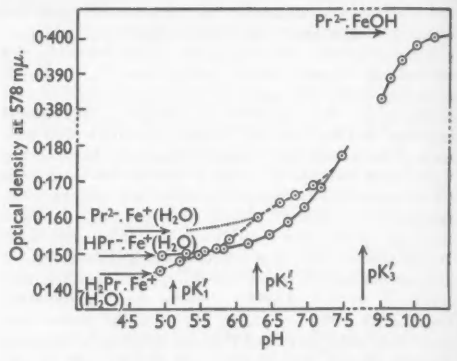

Fig. 3. Two titration curves of $4{\cdot}1 \times 10^{-5}$ M-MetHb plotting optical density at 578 mμ. against pH, at 20°. The continuous curve is at $I = 0{\cdot}26$ and shows the equilibrium between acid MetHb, the species $HPr^-.Fe^+(H_2O)$, and alk. MetHb, $Pr^{2-}.FeOH$. The dashed curve is at $I = 0{\cdot}01$ and shows the various species that appear below pH 7; it coincides with the other curve above pH 7·3. The dotted curve shows the extrapolation to the optical density corresponding to the species $Pr^{2-}.Fe^+(H_2O)$ calculated by the method described in the text.

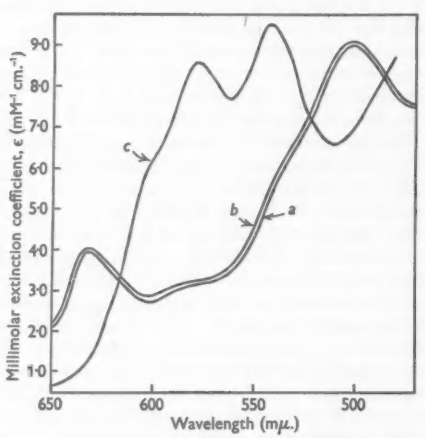

Fig. 2. Absorption spectra of MetHb at low and high pH, in the range 650–470 mμ. $T = 20°$, $I = 0{\cdot}02$. (*a*) Solution in phthalate buffer of pH 5·60. The curve was plotted after applying a slight extrapolation based on the known values of pK'_2 and pK'_3, and it corresponds to the species $HP^-.Fe^+(H_2O)$ of Eqns. 1 and 2. (*b*) Solution in phosphate buffer of pH 6·60. The curve represents maximum formation, about 80%, of the species $Pr^{2-}.Fe^+(H_2O)$ of Eqns. 2 and 3. (*c*) Solution in borate buffer of pH 10·2. The curve represents almost complete formation of alk. MetHb, which is the species $Pr^{2-}.FeOH$ of Eqn. 3.

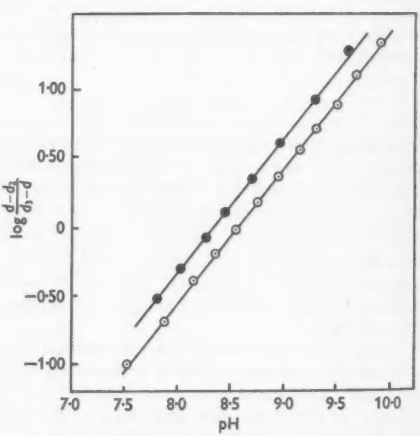

Fig. 4. Two tests of Eqn. 5 carried out at 20° using $5{\cdot}0 \times 10^{-5}$ M-MetHb in borate buffers. Values of $\log \frac{d-d_2}{d_3-d}$ are plotted against pH, the slopes of the lines being the values of n in Eqn. 5. Optical densities, d, were measured at 578 mμ.; d_3 is the corresponding optical density of 100% alk. MetHb and d_2 that of 100% acid MetHb obtained from an appropriate extrapolation. ⊙—⊙, test (1), $I = 0{\cdot}02$, $n = 1{\cdot}00$; ●—●, test (2), $I = 0{\cdot}22$, $n = 0{\cdot}99$.

where the brackets indicate molar concentration and where pH is measured against an arbitrary standard. However, the MetHb molecule contains four haematin groups which can ionize in this way, and if interaction occurs between these groups such that the ionization constant of one group is dependent on the degree of ionization of the others this would show itself by the experimental data fitting an equation of the type

$$\text{Const.} = n.\text{pH} + \log \frac{(\text{acid MetHb})}{(\text{alk. MetHb})}, \qquad (5)$$

where the value of n at 50 % reaction may be >1 or <1 depending on whether the ionization of one haematin group is favoured or hindered by the ionizations of the other haematin groups (cf. Wyman, 1948). Behaviour of this kind would be similar to that found in the combination of oxygen and carbon monoxide with reduced haemoglobin. Fig. 4 shows two runs which were carried out to test this point at two ionic strengths. The value of n was invariably found to be unity, which implies that no such interaction occurs and that the various haematin groups ionize independently. Eqn. 5 therefore reduces to Eqn. 4, and it was on this basis that pK'_2 values have been calculated. A different complication was encountered at low ionic strengths due to the appearance of three spectroscopic species of acid MetHb; a method of analysing the results at these low ionic strengths is described later.

It follows from the account given above that at ionic strengths which are conveniently high ($I > 0{\cdot}2$) the main ionization constant K'_3 may be calculated directly according to Eqn. 4. Details of the procedure are essentially the same as described in Paper 1. This method was found to be quite simple and accurate; mean deviations did not usually exceed ± 1 %. Several parallel runs also established that there were no specific effects due to the buffer or, excepting ammonium salts, to the added salt. Ammonium salts had a definite and reversible effect on the 578 mμ. band of alk. MetHb, similar to the effect of ammonia on alk. MetMb described by Theorell & Ehrenberg (1951).

The complications encountered at lower ionic strengths can be seen in Fig. 3. The shape of the curve suggests that there are three species involved in two overlapping ionizations with constants K'_2 and K'_3, as a consequence of which the middle species $Pr^{2-}.Fe^{+}(H_2O)$ is never completely formed; in fact it can be shown by analysing the data that its maximum formation corresponds to about 80 %. Similar considerations lead to the conclusion that the ionizations with constants K'_1 and K'_2 also overlap and hence that the species $HPr^{-}.Fe^{+}(H_2O)$ is also never fully formed. In view of these facts the determination of the three pK′ values requires the analysis of the data by a series of approximations. There is, however, another practical difficulty. The span, in units of optical density, of the pK'_2 ionization is only 4–5 % that of the main pK'_3 ionization, and so is that of the pK'_1 ionization. It is consequently difficult to obtain data accurate enough for the precise calculation of pK'_1 and pK'_2. On the other hand, pK'_3 can be obtained fairly easily by applying two successive approximations to the analysis of the results obtained in the usual type of run. This method of calculation was used extensively and the limits of error on pK'_3, even at the lowest ionic strengths, did not exceed ± 2 %. Fig. 5 shows the variation of pK'_3 with $\sqrt{I}$ at three temperatures, 20, 28 and 37°. The region of low ionic strengths has been investigated at 20° and 37° only.

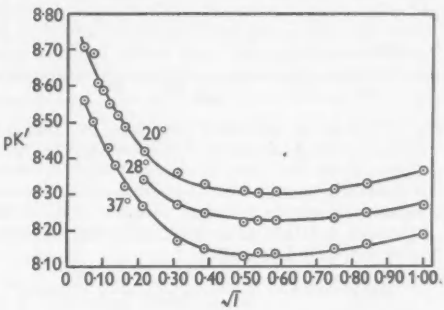

Fig. 5. The variation of pK'_3 for (horse) acid MetHb with ionic strength, I, and temperature. I is calculated from the contributions of the various buffer ions.

An alternative and more elegant method for the analysis of overlapping equilibria has been described recently (Thamer & Voigt, 1952). This method leads to the following relation between pK'_2 and pK'_3:

$$pK'_2 + pK'_3 = 2pH_m - \log \frac{d_m - d_2}{d_m - d_3}, \qquad (6)$$

in which d_m is the maximum optical density in the titration curve of Fig. 6 and pH_m is the corresponding pH value, d_2 and d_3 are the optical densities corresponding to 100 % species $HPr^{-}.Fe^{+}(H_2O)$ and $Pr^{2-}.FeOH$ (alk. MetHb) respectively. Of the terms in Eqn. 6 only pK'_3 and d_3 can be obtained with any certainty. Despite this severe limitation it was possible to obtain rough values of pK'_2 and to show that as the ionic strength increased from $I = 0{\cdot}01$ to $I = 0{\cdot}06$ the value of pK'_2 rose from about 6·33 to 6·40 at 20°. This effect is smaller and in the opposite sense to the effect of ionic strength on pK'_3. Fig. 6 shows one run at 22° and $I = 0{\cdot}016$ used for the calculation of a pK'_2 value together with the theoretical curve computed from the various parameters given in the legend.

No accurate method could be devised for the determination of pK_1', not only because of the difficulties outlined above but also due to the onset of irreversible changes below pH 4·6. Approximate estimates place pK_1' around 5·1 at 20° and $I = 0{\cdot}01$.

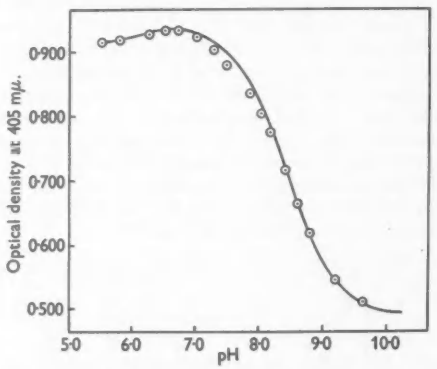

Fig. 6. Method of calculating pK_2' from the measured optical density at 405 mμ. as a function of pH. The experimental points are shown in circles. $T = 22°$, $I = 0{\cdot}016$. A theoretical curve, drawn as the continuous line, was calculated from the following parameters: $d_2 = 0{\cdot}916$, $d_m = 0{\cdot}933$, $d_3 = 0{\cdot}492$, $pH_m = 6{\cdot}70$, $pK_3' = 8{\cdot}48$, $pK_2' = 6{\cdot}34$ (after Thamer & Voigt, 1952).

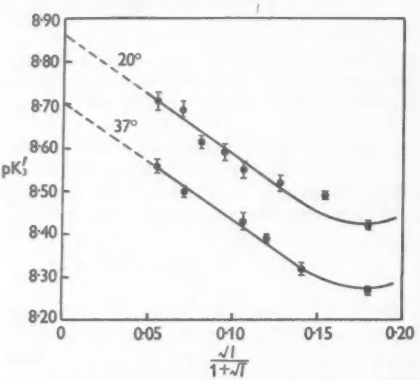

Fig. 7. Approximate thermodynamic ionization constants at 20° and 37° obtained from the data which are shown in Fig. 5 by a linear extrapolation of pK_3' values plotted against $\sqrt{I}/(1+\sqrt{I})$, where the ionic strength I includes the contributions of the buffer and salt ions as well as the contribution from MetHb estimated at the upper limit to be 0·001.

Approximate thermodynamic constants. Values for the approximate thermodynamic ionization constant K_3 were obtained by linear extrapolation to zero ionic strength. Fig. 7 shows the extrapolation at 20 and 37° made from $\sqrt{I}/1+\sqrt{I}$ plots. The two lines are approximately parallel with slopes of about $-2{\cdot}7$.

$$pK_3\,(20°) = 8{\cdot}86 \pm 0{\cdot}02, \quad pK_3\,(37°) = 8{\cdot}70 \pm 0{\cdot}02.$$

From these two values of pK_3 the corresponding heat of ionization ΔH may be calculated directly using the integrated van't Hoff isochore if one assumes that the value of ΔH is constant over the temperature range 20–37°. This gives

$$\Delta H^\circ = 3{\cdot}91 \pm 0{\cdot}49 \text{ kcal./mole.}$$

The corresponding free energy and entropy changes at, say, 20° may now be obtained:

$$\Delta G^\circ = -RT \ln K_3 = 11{\cdot}9 \pm 0{\cdot}03 \text{ kcal./mole,}$$

$$\Delta S^\circ = \frac{\Delta H^\circ - \Delta G^\circ}{T} = -27{\cdot}2 \pm 1{\cdot}8 \text{ cal./mole/degree.}$$

Methaemoglobin from different species. The results quoted so far have been those of studies made on horse MetHb. In view of the possibility that, at very small concentrations of the haemoprotein, neutral salts may cause the dissociation of horse MetHb molecules into smaller fragments (Gutfreund, 1949; see, however, Adair, 1949) extensive parallel studies were carried out on ewe MetHb, the molecules of which may have greater resistance to such dissociation. Substantially the same results were obtained, and the various spectral characteristics were present. The actual values of the ionization constant K_3' differed slightly, but consistently, from the values for horse MetHb. This behaviour is illustrated in Table 1 which gives some

Table 1. *A comparison of the ionization constant* K_3' *in horse and ewe MetHb over a range of ionic strength*

($T = 20°$. Method of calculating pK_3' is given in the text. I = ionic strength calculated from the contributions of buffer and salt ions. The estimated probable errors are shown in brackets.)

I	pK′ horse (±0·01)	pK′ ewe (±0·02)	$\frac{\text{K′ ewe}}{\text{K′ horse}}$ (±0·08)
0·006	8·66	8·62	1·10
0·029	8·47	8·41	1·15
0·050	8·41	8·34	1·18
0·123	8·34	8·28	1·15
0·155	8·33	8·26	1·18
0·304	8·30	8·24	1·15

pK_3' values at 20° for both species of haemoglobin. No difference in behaviour attributable to protein dissociation could be detected in this comparative study.

DISCUSSION

A comparison of the results with those of Paper I shows that the main ionization of (horse) acidic methaemoglobin, with ionization constant K_3', is similar in many respects to the ionization of (horse)

acidic metmyoglobin. The first important conclusion is the confirmation that the ionizations of the four haematin-bound water molecules in MetHb are single and independent. There are two lines of evidence for this. The similar behaviour of horse and sheep MetHb, with their varying degrees of dissociation into subunits, supports the view that the ionizations of the haems within the molecule are independent. Moreover, the value of n in Eqn. 5 was invariably found to be unity. This shows that the ionization involves one H^+ per Fe atom, and that pK_3' is constant over the pH range 7·8–10·0 even at a low ionic strength. There is no 'tailing' of pK_3' values, as was encountered in the case of the MetMb ionization, and hence there is no reason to suppose that any other equilibria affect the ionization of the iron-bound water molecule in acid MetHb above pH 9.

It has also been shown that both ionizations are endothermic processes, on which neutral salts have the same marked effect, though with MetHb the reversal of this effect is more easily observed because it occurs at a relatively higher ionic strength ($I \simeq 0{\cdot}1$ for MetHb and 0·01 for MetMb). Further, the limiting slope of the plots of pK_3' against $\sqrt{I}/(1+\sqrt{I})$, is about $-2{\cdot}7$ which, according to a simple Debye-Hückel model, indicates that acid MetHb has the charge type -2 and alk. MetHb -3. Hence the effective negative charge on MetHb, influencing the ionization of the iron-bound water, must be small even in solutions 3 pH units on the alkaline side of the isoelectric point. It is for this reason that its contribution to ionic strength has been taken at the upper limiting value of 0·001. A similar conclusion about MetMb was arrived at in Paper 1.

It is interesting to note that other workers have also arrived at similar conclusions. Thus Stadie (1928) examined the effect of haemoglobin on the activity coefficient of HCO_3^- ions and concluded that its 'ionic strength valence' was about unity. Barnard (1944) carried out acid-base titrations of canine haemoglobin and concluded that its ionic strength valence was zero at the isoelectric point, and that it rose on either side of this point, reaching a maximum of -3 in alkaline solutions. Clearly, these charge types can only accord with a Debye-Hückel model in solutions of extremely low ionic strength. However, Theorell & Ehrenberg (1951) as well as Coryell *et al.* (1937) and Wyman & Ingalls (1941) have all used linear extrapolation of pK′ values from the region of high ionic strength with the result that their 'extrapolated' ionization constants are as much as 8 times the value of the true thermodynamic constants. For instance the above results give pK_3 (25°) = 8·80, compared with the value 7·89 obtained by Coryell *et al.* (at, presumably, 24 ± 2°).

The approximate thermodynamic constants given in this paper are subject to the same limits of uncertainty mentioned in Paper 1. When the two sets of data are compared (at, say, 20°) a curious compensation of the heat and entropy terms can be seen. Thus for MetHb, $\Delta H = 3{\cdot}91 \pm 0{\cdot}49$ kcal./mole and $\Delta S^\circ = -27{\cdot}2 \pm 1{\cdot}8$ cal./mole/degree, for MetMb $\Delta H^\circ = 5{\cdot}75 \pm 0{\cdot}67$ kcal./mole and

$$\Delta S^\circ = -21{\cdot}7 \pm 2{\cdot}4 \text{ cal./mole/degree.}$$

Consequently, the corresponding free energies, and hence the thermodynamic ionization constants, are close to each other, the values of pK at 20° being $8{\cdot}86 \pm 0{\cdot}02$ for MetHb and $9{\cdot}04 \pm 0{\cdot}03$ for MetMb.

In these spectrophotometric studies the main difference between the behaviour of MetHb and that of MetMb has been the detection, in the former, of two ionizations possessing a haematin-linked property and characterized by equilibrium constants K_1' and K_2'. The term 'haematin-linked property' has been used rather than 'haematin-linked group' because the present data only reveal a change in the spectroscopic properties of the molecule. The very nature of this chemical reaction of the iron atom precludes a direct determination of its equilibrium constant K_3' in the pH range 5–7 where the ionizations indicated in Eqns. 1 and 2 occur. No evidence can therefore be obtained to show whether the affinity of the haematin iron for a proton depends on the extent of ionization of these groups which would then justify the use of the term 'haematin-linked group' (Wyman, 1948).

The presence of two haematin-linked ionizations in horse MetHb has been deduced previously from other studies. Coryell & Pauling (1940) concluded from a magnetic titration of MetHb that $pK_1' = 5{\cdot}3$; Theorell (1943) gave the value 5·45 at 20° and low ionic strength based on an acid-base titration of MetHb, and Wyman & Ingalls (1941) obtained 5·17 at 30° and $I = 0{\cdot}16$ from an analysis of a differential acid-base titration of MetHb and HbO_2 and a consideration of other related data. In this paper the approximate value of pK_1' is estimated to be 5·1 at 20° and $I = 0{\cdot}01$ and that of pK_2' to be 6·34 at 20° and $I = 0{\cdot}016$. The latter value presumably corresponds to pK′ = 6·65 at 30° and $I = 0{\cdot}2$ deduced by Taylor & Hastings (1939) from the effect of pH on the oxidation-reduction potential of the Hb-MetHb system, and to pK′ = 6·50 at 20° and low ionic strength obtained by Theorell (1943) from the acid-base titration of MetHb. In view of the uncertainties about the heats of ionization and the effects of neutral salts on pK_1' and pK_2' no thorough comparison between the various results can be made, but it is worth noting that in these spectrophotometric studies the value of pK_2' has been shown to rise with increasing ionic strength, and so the discrepancies between the results are probably much less than they appear to be.

The structural interpretation of the relation between these two ionizations and the haematin iron must now also explain the observation that neutral salts can modify their haematin-linked properties in such a way that, at high ionic strengths, the absorption spectrum of acid MetHb is also that of the conjugate base of the ionization in Eqn. 1 (with equilibrium constant K_1') and that of the conjugate acid of the ionization in Eqn. 2 (with constant K_2').

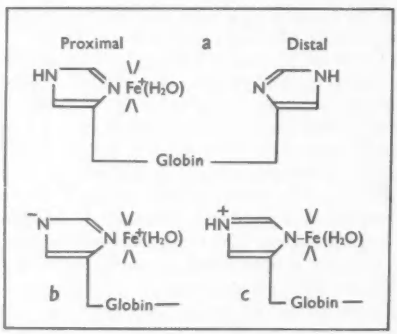

Fig. 8. Configurations of the haematin group in MetHb chosen to illustrate the possible effect of neutral salts on haematin-linked ionizations. (*a*) Schematic representation in which the iron atom is situated between the glyoxalinium N atoms of two histidine groups, with a strong bond to the proximal group and a weak bond to the distal group (after Coryell & Pauling, 1940), but the bonds are not specified in the diagram. (*b*) Proximal histidine with the imino N in the form of its conjugate base, representing one particular electronic configuration of the glyoxaline ring. (*c*) An alternative electronic configuration of the glyoxaline ring with the imino N in the form of its conjugate acid.

The type of interaction which can account, in part, for this neutral salt effect may be illustrated with reference to the structural model developed by Wyman (1939) and Coryell & Pauling (1940). In this model, which is shown in Fig. 8 (*a*), the iron atom is firmly bound to the glyoxalinium nitrogen atom of a histidine on one side of the haematin plane and is near enough for weak interaction to occur with another histidine group on the other side of the plane. The haematin-linked ionizations are attributed to the imino nitrogen of the first, or proximal, glyoxaline group and to the glyoxalinium nitrogen of the second, or distal, group. If the proximal group has the electronic configuration shown in Fig. 8 (*b*), then the clustering of cations, from a neutral salt, could reduce the electrostatic effect of the negatively charged base such that the spectrum resembles that of the conjugate acid. Conversely, if this group has the electronic configuration in Fig. 8 (*c*), then the clustering of salt anions could reduce the electrostatic effect of the positively charged acid group such that the spectrum resembles that of the conjugate base. This ionization can therefore be in accord with the salt effect on either pK_1' or pK_2'. However, the ionization of the distal group can accord with the salt effect in one sense only. The interaction between this group and the iron occurs only when the group is in its basic form and is prevented when salt cations shield the glyoxalinium nitrogen atom, in which case the spectrum would resemble that of its conjugate acid.

Coryell & Pauling (1940) attributed pK_1' to the glyoxalinium nitrogen of the distal group and pK_2' to the imino nitrogen of the proximal group. The above discussion shows that the effect of neutral salts on these two ionizations is in agreement with either pK_1' or pK_2' being attributed to the proximal group but not with pK_1' involving interaction between the iron and the distal group. In any structure where bonding to the iron involves an atom which is a proton acceptor this disagreement persists. Further, a water molecule must be incorporated in the methaemoglobin structure, with possibly an O—H...N hydrogen bond to the distal glyoxaline group. Although such a model is more acceptable than that in Fig. 8 (*a*) it does not help to resolve the difficulty.

It is structurally possible for the propionic acid side chain to be near enough to any proximal group or to the iron-bound water molecule for some type of weak bonding to occur, but there appears to be no particular reason for choosing such structures. Besides, arguments similar to those given above also lead to uncertainties about the identification of a pK′ value with a particular dissociating group. Independent knowledge of the charge types involved in these ionizations would help enormously to clarify this problem, as would a knowledge of the chemical nature of these groups.

SUMMARY

1. The spectrophotometric acid-base character of horse methaemoglobin has been investigated and compared with that of horse metmyoglobin.

2. All the results are consistent with the view that the ionizations of the four haematin-bound water molecules in methaemoglobin are single and independent. There is no evidence for interaction effects between the various haem groups and, unlike the case of metmyoglobin, no other ionizing groups appear to affect this ionization above pH 9.

3. The ionization of acid methaemoglobin, like that of acid metmyoglobin, shows a marked neutral-salt effect which also indicates that its 'ionic strength valence' per haem does not exceed -3 even at pH 10.

4. Approximate thermodynamic constants for this ionization have been determined by extrapolation from the data obtained at the lowest ionic strengths. The ionization constant

$$pK_3\,(20°) = 8{\cdot}86 \pm 0{\cdot}02,$$
$$\Delta H = 3{\cdot}91 \pm 0{\cdot}49 \text{ kcal./mole},$$
$$\Delta S°\,(20°) = -27{\cdot}2 \pm 1{\cdot}8 \text{ cal./mole/degree}.$$

The values of pK_3 which previous workers obtained by extrapolation from the high ionic strength region are considerably different from the values given in this paper.

5. The spectral characteristics of ewe methaemoglobin are very similar to those of horse methaemoglobin. In a comparative study of the ionization the values of the ionization constant K_3' were found to be substantially the same in the two species, and no differences attributable to possible dissociation of the haemoprotein into subunits could be detected.

6. Small reversible changes in the absorption spectrum of acid methaemoglobin, not hitherto observed, have been attributed to two ionizations, probably of haematin-linked groups. At 20° and $I = 0{\cdot}01$ the following approximate values for these ionization constants have been obtained: $pK_1' = 5{\cdot}1$, $pK_2' = 6{\cdot}33$.

7. Spectroscopically, these two ionizations are remarkably sensitive to neutral salts. At comparatively high ionic strengths ($I > 0{\cdot}2$) no change can be detected in the spectrum of acid methaemoglobin over the pH range where these ionizations occur. The effect is such that, at all values of $I > 0{\cdot}2$, the spectrum of acid methaemoglobin corresponds to the conjugate acid of pK_2', which is identical with the conjugate base of pK_1'.

8. The effect of neutral salts on the haematin-linked ionizations is discussed with reference to possible structures for methaemoglobin.

We are much indebted to Prof. D. Keilin, F.R.S., for providing facilities in the Molteno Institute for the preparation of the haemoproteins used in these investigations and wish to record our thanks to Dr E. F. Hartree for his advice and help with the methods employed. Our thanks are also due to the Medical Research Council for a grant to one of us (G. H.).

REFERENCES

Adair, G. S. (1949). *Haemoglobin*, p. 191. London: Butterworth.
Austin, J. H. & Drabkin, D. L. (1935). *J. biol. Chem.* **112**, 67.
Barnard, R. D. (1944). *J. biol. Chem.* **153**, 91.
Coryell, C. D. & Pauling, L. (1940). *J. biol. Chem.* **132**, 769.
Coryell, C. D., Stitt, F. & Pauling, L. (1937). *J. Amer. chem. Soc.* **59**, 633.
Duve, C. de (1948). *Acta chem. scand.* **2**, 264.
Gamgee, A. (1868). *Phil. Trans.* **158**, 589.
George, P. & Hanania, G. (1952). *Biochem. J.* **52**, 517.
Gutfreund, H. (1949). *Haemoglobin*, p. 197. London: Butterworth.
Hartridge, H. (1920). *J. Physiol.* **54**, 253.
Haurowitz, F. (1924). *Hoppe-Seyl. Z.* **138**, 68.
Haurowitz, F. (1949). *Haemoglobin*, p. 53. London: Butterworth.
Hicks, C. S. & Holden, H. F. (1929). *Aust. J. exp. Biol. med. Sci.* **6**, 175.
Horecker, B. L. (1943). *J. biol. Chem.* **148**, 173.
Keilin, D. & Hartree, E. F. (1935). *Proc. Roy. Soc.* B, **117**, 1.
Keilin, D. & Hartree, E. F. (1949). *Nature, Lond.*, **164**, 254.
Keilin, D. & Hartree, E. F. (1951). *Biochem. J.* **49**, 88.
Keilin, D. & Hartree, E. F. (1952). *Nature, Lond.*, **170**, 161.
Stadie, W. C. (1928). *J. biol. Chem.* **77**, 303.
Taylor, J. F. & Hastings, A. B. (1939). *J. biol. Chem.* **131**, 649.
Thamer, B. J. & Voigt, A. F. (1952). *J. phys. Chem.* **56**, 225.
Theorell, H. (1943). *Ark. Kemi Min. Geol.* **16A**, no. 14.
Theorell, H. & Ehrenberg, A. (1951). *Acta chem. scand.* **5**, 823.
Wyman, J. jun. (1939). *J. biol. Chem.* **127**, 581.
Wyman, J. jun. (1948). *Advanc. Protein Chem.* **4**, 439.
Wyman, J. jun. & Ingalls, E. N. (1941). *J. biol. Chem.* **139**, 877.

The Amino Acids and other Ampholytes of Urine

2. THE ISOLATION OF A NEW SULPHUR-CONTAINING AMINO ACID FROM CAT URINE

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The application of two-way paper chromatography to the study of the amino acids excreted in human urine (Dent, 1947) has facilitated the recognition of the amino acidurias which are associated with certain pathological conditions. The method has now been applied for several years and a large number of urine samples from normal and pathological subjects has been examined (Dent & Harris, 1951; Crumpler, Dent, Harris & Westall, 1951). Datta & Harris (1951) also investigated the urinary amino-acid patterns of other mammals and it was apparent from this study that there were wide variations in the pattern of amino-acid excretion to be observed between different species. This finding was, perhaps, not unexpected in view of the different diets involved. It was, however, surprising to find that a ninhydrin-reacting substance excreted by the domestic cat in quantity and by the ocelot in lesser amount was a hitherto undescribed amino acid.

This substance, now named 'felinine' but referred to as 'cat-spot' by Datta & Harris (1951), partially overlays leucine and isoleucine on the phenol-ammonia collidine-lutidine two-way paper chromatogram, but it can be readily separated from them on a one-way chromatogram run with *tert.*-amyl alcohol. After oxidation with hydrogen peroxide (Dent, 1948) the spot no longer appeared in its original position on two-dimensional chromatograms but in the presence of other amino acids it was difficult to decide whether it had moved to a new position or whether it had been degraded to such an extent that it failed to give a ninhydrin colour. However, the suspicion remained that felinine was a new sulphur-containing amino acid. The following sulphur-containing amino acids were run on two-way chromatograms: homocysteine, homomethionine, lanthionine, $\beta\beta$-dimethylcysteine, $\beta\beta\beta'\beta'$-tetramethylcystine and the series of normal C_1—C_5 *S*-alkylcysteines. However, the ninhydrin-sprayed spots due to these substances failed, in every instance, to match the position of felinine.

The pure amino acid has now been isolated from cat urine by fractionation on ion-exchange columns (Westall, 1952). Felinine was recovered from the primary fractionation on Zeo-Karb 215 mixed with other amino acids. Treatment of this mixture on columns of the anion-exchange resin Dowex 2 yielded fractions containing felinine contaminated only with a small proportion of another unknown amino acid designated as 'peach-spot' (on account of its ninhydrin colour reaction) and which moves to a position just above glutamic acid in the diagram of Dent (1948). Felinine and 'peach-spot' could not be separated by fractionation on either Zeo-Karb 215 or Dowex 2 resin columns at room temperature, but separation was achieved on a Zeo-Karb 215 column operated at 65° (Partridge & Brimley, 1951). Under these conditions the 'peach-spot' substance was largely degraded and a decomposition product was found in the early fractions whilst felinine was recovered from the later fractions.

The amino acid has not been crystallized. It was readily soluble in water and an aqueous solution, on evaporation, formed a syrup which could not be induced to crystallize. Addition of ethanol in small amounts gave a clear thixotropic gel and further additions yielded no crystals. An opaque white gelatinous precipitate was formed on addition of ether to the ethanol solution, but this material had no apparent crystalline structure. Precipitation with acetone from the aqueous-ethanolic solution provided the best method of isolating the substance. It is well known that small amounts of impurities inhibit the crystallization of certain amino acids but, in this case, such an explanation would appear unlikely. Samples from three separate isolations of the amino acid from different collections of cat urine have all yielded analyses which agree within very narrow limits.

The simplest empirical formula that could be derived was $C_8H_{17}O_3NS$ (mol.wt. 207) and a determination of the molecular weight (240) by freezing-point depression in water indicated that this formula was reasonable. Since this substance was retained (in quantity) by both cation- and anion-exchange resins it was inferred that it possessed a basic group and at least one acidic group. The total nitrogen (Dumas) could be entirely accounted for as NH_2-N by the nitrous acid method and an equivalent of carbon dioxide was liberated using the ninhydrin-carbon dioxide method with a citrate

buffer at pH 2·5. The material when run on the two-way chromatograms in the presence of copper carbonate (Crumpler & Dent, 1949) formed a copper complex and failed to give a ninhydrin spot. It was thus reasonable to suppose that the substance was an α-amino acid.

Oxidation of the amino acid

As the felinine contained sulphur, the change in position on a chromatogram of the ninhydrin spot after treatment with hydrogen peroxide was probably due to oxidation of a sulphur atom. Fig. 1

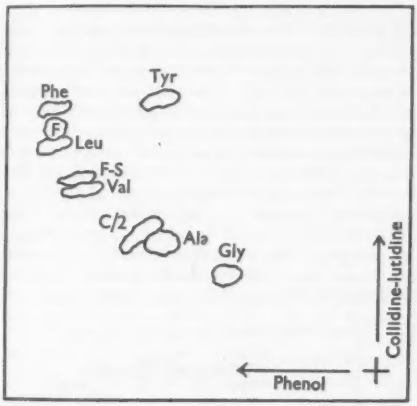

Fig. 1. A two-way paper chromatogram showing the relative positions of felinine and its derivatives. Ala, alanine; C/2, C/2 substance; F, felinine; F-S, felinine sulphone; Gly, glycine; Leu, leucine; Phe, phenylalanine; Val, valine; Tyr, tyrosine.

shows the position of its oxidation product in relation to several other amino acids. The substance was partly oxidized by air during chromatography, and faint spots were frequently observed on the chromatograms after treatment with ninhydrin. One of these spots appeared to be due to oxidation during the drying off of phenol before rerunning in the collidine-lutidine solvent mixture, whilst another could be due to the formation of a sulphoxide. A compound $C_8H_{17}O_5NS$ formed by oxidation of felinine with hydrogen peroxide has been isolated. Analysis indicated that the original substance had taken up two atoms of oxygen with the probable formation of a sulphone. This idea was supported by the observation that equimolar quantities of felinine and methionine took up the same amount (2·2 atoms/mol.) of oxygen from potassium permanganate. The oxidized derivative decomposed vigorously at 120–125° and slowly darkened on storage at room temperature.

Treatment with Raney nickel

Distillation of an aqueous solution of felinine in the presence of Raney nickel yielded water, together with a small quantity of a colourless liquid which formed an upper layer. This substance was identified as *iso*amyl alcohol by formation of a 3:5-dinitrobenzoate and by oxidation with dilute chromic acid to *iso*valeraldehyde which gave a crystalline derivative with 2:4-dinitrophenylhydrazine. Alanine was isolated from the reaction residue and proved to be the only ninhydrin-reacting substance present. From the evidence gained in the reaction with Raney nickel it is reasonable to assume that felinine contains cysteine linked to *iso*amyl alcohol by its sulphur atom (I). This structure is provisional.

$$HO_2C.CH(NH_2).CH_2.S.C(CH_3)_2.CH_2.CH_2OH \quad (I)$$

Degradation of the amino acid

When felinine was heated with concentrated hydrochloric acid some of the original substance was found unchanged, together with traces of cystine and cysteine, whilst a second unknown sulphur-containing amino acid was produced in fair yield (approx. 40 %). A brown oily substance was also liberated. The amino acids were fractionated first on a column of Zeo-Karb 215 and then on a column of Dowex 2 and the hydrolysis product (C/2) crystallized. Analyses gave the empirical formula $C_{11}H_{22}O_4N_2S_2$. When run on a two-way paper chromatogram using phenol-ammonia and collidine-lutidine the C/2 substance moved to a position close to alanine (Fig. 1). No ninhydrin-colouring spot was observed in this solvent system after oxidation of the substance with hydrogen peroxide. The oxidation product decomposes in phenol but it survived after running on paper with a butanol-acetic acid mixture (Partridge, 1948) and had R_F 0·10. Treatment of an aqueous solution of the C/2 substance with Raney nickel gave alanine in good yield as the only surviving ninhydrin-reacting substance.

Treatment of felinine with 2 N-sodium hydroxide at 100° destroyed the amino acid and no substance remained which would yield a ninhydrin-reacting spot. All the nitrogen, presumably as ammonia, was trapped in acid when the substance was distilled in the presence of 2 N-sodium hydroxide.

In an attempt to explain the series of degradations which occur when felinine is hydrolysed with acid the following scheme, based on the assumption that formula I represents the general structure, is provisionally suggested.

Under the treatment with acid some of the felinine breaks down with the liberation of free cysteine (II). The cysteine then condenses with the unchanged felinine eliminating water and forming a di-cysteine derivative (C/2) (III). This derivative would have

the found empirical formula ($C_{11}H_{22}O_4N_2S_2$) and would liberate alanine and *iso*pentane on treatment with Raney nickel. Whilst the formation of alanine has been confirmed, it has not, so far, been possible to characterize the hydrocarbon. However, some further evidence for the above scheme was provided by increased production of the dicysteine substance by the addition of L-cysteine to the mixture before hydrolysis. The production of a brown oil during the treatment with acid probably arose by polymerization of the C_5 residue remaining after the liberation of free cysteine. The identity of this substance is unknown, but analysis has shown that it contained neither nitrogen nor sulphur.

$$HO_2C.CH(NH_2).CH_2.S.CMe_2.CH_2.CH_2OH \quad (I)$$

$$\downarrow$$

$$(II) \quad HO_2C.CH(NH_2).CH_2SH + CH_2{:}CMe.CH_2.CH_2OH \rightarrow \text{Polymer}$$

$$\downarrow +(I)$$

$$(III) \quad HO_2C.CH(NH_2).CH_2.S.CMe_2.CH_2.CH_2.S.CH_2.CH(NH_2).CO_2H + H_2O.$$

EXPERIMENTAL

M.p.'s are given uncorrected.

Cat urine (5·2 l.) was concentrated under reduced pressure at 50° to 1 l. Ethanol (3 l.) was added and the mixture was left at 0° overnight. The precipitate was removed and washed with 80% (v/v) ethanol and the filtrate and washings were evaporated to a syrup. Water (2 l.) was added and this solution was shaken with 100 g. of charcoal powder (previously washed with 10% (v/v) aqueous acetic acid and then with water). The charcoal was removed by filtration and the clear solution, diluted to 10 l. with water, was run through a system of large columns containing the cation-exchange resin Zeo-Karb 215. The details of these columns and the method used for their operation were described in Part I of this series of papers (Westall, 1952). The amino acids were displaced with 0·25 N-ammonia and 40 fractions (each 50 ml.) were collected. The amino-acid composition of these fractions, determined by paper chromatography, is illustrated in Fig. 2. It will be observed that felinine occurred in fractions 5–20 (inclusive) along with a number of other amino acids. These fractions were bulked (800 ml.), boiled for 1 min. to expel CO_2 and transferred to a series of columns containing the anion-exchange resin Dowex 2. The amino acids were displaced with 0·1 N-HCl and collected in 55 fractions (25 ml. each). The amino-acid composition of these fractions is illustrated in Fig. 3. Fractions 27–45 contained felinine contaminated only with a trace of an unknown substance which gave a peach-coloured ninhydrin spot on the paper chromatograms. These two substances formed mixed bands when fractionated on both the Zeo-Karb 215 and Dowex 2 columns. However, a separation was achieved by using a heated Zeo-Karb 215 column (Partridge & Brimley, 1951). The series of small columns was jacketed and water at 65° was circulated continuously. The mixture (fractions 27–45 bulked) was applied to the columns in the usual way and the amino acids were displaced with 0·1 N-ammonia. Under these conditions the 'peach-spot' substance was concentrated in the early fractions, whereas the later fractions contained felinine only. Thirty fractions (each 25 ml.) were collected and nos. 5–28 were selected for the isolation. These fractions were combined and evaporated under reduced pressure to a pale-yellow syrup. Ethanol (10 ml.) was added, followed by 250 ml. of acetone which was added gradually with occasional shaking. After leaving overnight at 0° the white flocculent precipitate was filtered, washed with acetone and dried over H_2SO_4; yield 6·25 g., m.p. 177°, from 5·2 l. of cat urine. (Found: C, 46·2; H, 8·2; N (Dumas), 6·7; S, 15·4%. $C_8H_{17}O_3NS$ requires C, 46·4; H, 8·2; N, 6·8; S, 15·4%. Amino N (Van Slyke nitrous acid), 6·8%; carboxyl N, 6·7%; $[\alpha]_D^{20}$ −23° (*c*, 2·2 in water).)

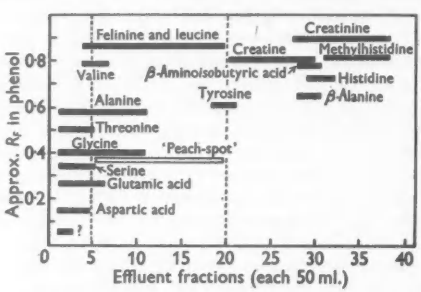

Fig. 2. Primary fractionation of cat urine on Zeo-Karb 215. Displacement chromatography using ammonia.

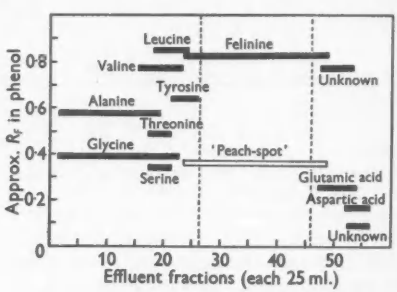

Fig. 3. Secondary fractionation on Dowex 2. Displacement chromatography using HCl.

Preparation of felinine sulphone

To 0·20 g. felinine in 25 ml. of water was added 2 ml. of 30% (v/v) H_2O_2 and the mixture was left at room temperature for 30 min. The solution was evaporated under reduced pressure at 40° to 10 ml. in order to remove excess H_2O_2. Paper-chromatographic analysis indicated that the original felinine had reacted completely. The aqueous solution was evaporated to a syrup and, after the addition of 1 ml. ethanol, a white flocculent precipitate was obtained by the gradual addition of 20 ml. of acetone. Yield 0·15 g.

m.p. 120–125° (decomp.). (Found: C, 40·7; H, 7·1; N (Dumas), 5·6; S, 12·9%. $C_5H_{11}O_5NS$ requires C, 40·2; H, 7·1; N, 5·8; S, 13·4%.)

Treatment with Raney nickel

Felinine (0·5 g.) was dissolved in 5 ml. water in a micro-distillation flask and 1 g. of Raney nickel (Bougault, Cattelain & Chabrier, 1938) was added, after 1 hr. the mixture was distilled and the first few drops of distillate were collected. Two layers were observed and the upper layer (alcohol) was transferred by means of a micropipette to a dry tube. Successive additions of about 0·2 g. of Raney nickel were made followed by distillation until the distillate failed to give two layers.

Isolation of alanine. The aqueous solution from the above experiment was filtered free from Raney nickel and transferred to a small column of Zeo-Karb 215. The column was washed with water and 0·1 N-NH_3 was applied to displace the alanine. As soon as a positive ninhydrin reaction was obtained on the effluent, fractions (approx. 5 ml.) were taken and the collections proceeded until the breakthrough of ammonia. Paper-chromatographic analysis of these fractions showed that alanine was the only amino acid present. The fractions were combined, evaporated to 3 ml. and the alanine was crystallized by addition of 3 vol. of acetone. Yield, 0·55 g. (72% of theoretical). (Found: N (Dumas), 15·6%. Calc. N, 15·73%.)

Preparation of the 3:5-dinitrobenzoate of the alcohol. 1 ml. of a solution of 1 g. 3:5-dinitrobenzoyl chloride (in 2 ml. ligroin + 8 ml. dry benzene) and 0·5 ml. of pyridine were added to the tube containing the unknown alcohol. The mixture was refluxed on a water bath for 30 min. After cooling, the mixture was treated in the usual manner and the resulting crystals after recrystallization from ethanol-ether (4:1, v/v) had m.p. 63·5°. A derivative prepared similarly from *iso*amyl alcohol gave m.p. 64°; mixed m.p. 63·5°.

Preparation of the 2:4-dinitrophenylhydrazone of the aldehyde obtained by mild oxidation of the alcohol. An aeration train of four tubes was connected in series: the first tube contained conc. H_2SO_4, the second 0·5 g. Raney nickel and 0·05 g. felinine in 1·5 ml. water, the third 1·5 ml. 2 N-H_2SO_4 with a small crystal of $K_2Cr_2O_7$ and the last tube contained 2 ml. of a saturated solution of 2:4-dinitrophenylhydrazine in 2 N-HCl. A gentle stream of air was passed through the solutions and the tube which contained dilute chromic acid was kept at 50°. After allowing aeration to continue for 1 hr., the orange-coloured precipitate which formed in the last tube was filtered off and recrystallized from aqueous ethanol (m.p. 122·5°). *iso*Valeraldehyde 2:4-dinitrophenylhydrazone prepared similarly had m.p. 123°. Mixed m.p., 122·5°.

Action of acid on felinine and isolation of substance C/2

The amino acid (0·5 g.) was heated with 10 ml. 5 N-HCl in a sealed tube at 105° for 36 hr. 50–100 µl. of a brown oil separated out on the surface. Its composition is at present unknown but N and S were shown to be absent. After removing the oil, the solution was evaporated under reduced pressure and the resulting syrup was taken up in water (100 ml.). This solution was transferred to a column of Zeo-Karb 215 resin (two tubes in series; diam. 1·5 and 0·9 cm.; contents 8 and 2 g. resin respectively). The column was washed with water to remove the remaining traces of HCl from the hydrolysate and the amino acids were displaced with 0·12 N-NH_3. The effluent was collected in nine fractions (each 5 ml.). Paper-chromatographic analysis of these fractions showed that only a partial separation of the component amino acids had been obtained. These fractions, now free of HCl, were combined and transferred to a set of columns of Dowex 2 (two tubes in series; diam. 1·2 and 0·7 cm.; contents 5 and 1·5 g. of resin respectively). The amino acids were displaced with 0·05 N-HCl and nine fractions (each 5 ml.) were collected. Fractions 1–4 contained unchanged felinine, 2–9 the C/2 substance, whilst cystine and cysteine were present in 8 and 9. Fractions 4–7, which apparently contained C/2 substance only, were combined (20 ml.) and evaporated to 2 ml. A crop of white crystals was obtained. Yield, 103 mg.; m.p. 230–232°. (Found: C, 43·1; H, 7·1; N (Dumas), 9·0; S, 21·0%. $C_{11}H_{22}O_4N_2S_2$ requires C, 42·6; H, 7·1; N, 9·05; S, 20·6%.)

DISCUSSION

The urine used for this and other isolations was collected from small colonies of cats kept in metabolic cages but samples of urine from cats kept under domestic conditions have also been examined by two-way chromatograms and these have shown similar urinary patterns. Felinine is usually the most abundant amino acid excreted. The 24 hr. output (approx. 100 ml.) may contain as much as 100–120 mg. of the substance. Examination of cat blood-plasma ultrafiltrate by two-way paper chromatograms has shown no trace of felinine, but this evidence alone is insufficient to rule out the probability of its presence in low concentration.

The significance of the occurrence of this sulphur amino acid in cat urine is at present unknown. It is improbable that it occurs as such in the diet, as the amount excreted is relatively high; moreover, as the food given to the cats is similar to that consumed by man, it is unlikely that the amino acid would have escaped detection in these foods. Even so, the type of diet may have some influence on the excretion of felinine since in one pair of cats transferred from a mixed diet to one of fish only there was a marked decrease in the excretion of felinine with a simultaneous increase in the taurine output (Datta, S. P. & Harris, H., unpublished). In fermentation by yeast, *iso*amyl alcohol is known to arise from leucine by deamination and decarboxylation. In rats, Bloch (1944) found that, after feeding leucine or *iso*valeric acid labelled with deuterium, he obtained identical isotopic concentrations in the tissue cholesterol. From this he concluded that *iso*valeric acid is an intermediate in leucine metabolism. It is possible therefore that *iso*amyl alcohol occurs in the series of reactions leading from leucine to *iso*valeric acid. In cats, a partial block in this mechanism might lead to an accumulation of *iso*amyl alcohol which, being toxic, would probably conjugate with some other substance before being excreted.

Cysteine would be available for this reaction and felinine might be the non-toxic end product. On the other hand the new amino acid could be a normal product of mammalian metabolism and take part in the transfer of C_5 units (i.e. isoprene-type) or C_3 units (*iso*propyl units found by oxidation and removal of C_2 as acetate) in the synthesis of such substances as sterols or carotenes which require a branched-chain carbon skeleton. Whilst such a substance need, in most animals, occur only transiently, it is possible that in the cat, owing to some specific peculiarity, this substance is accumulated and excreted.

The C/2 substance which is formed on treatment with acid is not believed to occur naturally. No trace of it was observed on the two-way paper chromatograms of cat urine and none was encountered in the course of the large-scale fractionation.

The structure proposed for felinine must remain provisional until further proof becomes available. Some attempts have already been made to synthesize the new amino acid and also the C/2 substance derived from it. This work will be reported in a later paper.

SUMMARY

1. A new sulphur-containing amino acid (felinine) has been isolated from cat urine following a series of fractionations on ion-exchange resins.

2. This substance is excreted by the cat in amounts as high as 100–120 mg./day, which is greater in amount than that of any other single amino acid.

3. Degradative studies with Raney nickel led to the identification of alanine, *iso*amyl alcohol and hydrogen sulphide indicating that felinine is a *S*-hydroxyalkylcysteine, the alkyl group having the carbon skeleton of *iso*pentane.

4. On treatment with mineral acid felinine gives rise to a second sulphur-containing amino acid (C/2), which is believed to be a dicysteine derivative. This substance does not occur as such in cat urine.

The author wishes to record his thanks to Prof. F. Challenger and Dr C. E. Dent for their advice and interest during the course of this work. The micro-analyses for C, H, N and S were carried out by Weiler and Strauss, Oxford. I should also like to thank Dr S. P. Datta of the Biochemistry Department, University College, London, and Dr E. Reid of the Biochemistry Department, Cambridge, for supplies of cat urine.

REFERENCES

Bloch, K. (1944). *J. biol. Chem.* **155**, 255.

Bougault, J., Cattelain, E. & Chabrier, P. (1938). *Bull. Soc. chim. Fr.* [5], **5**, 1699.

Crumpler, H. R. & Dent, C. E. (1949). *Nature, Lond.*, **164**, 441.

Crumpler, H. R., Dent, C. E., Harris, H. & Westall, R. G. (1951). *Nature, Lond.*, **167**, 307.

Datta, S. P. & Harris, H. (1951). *J. Physiol.* **114**, 39*P*.

Dent, C. E. (1947). *Biochem. J.* **41**, 240.

Dent, C. E. (1948). *Biochem. J.* **43**, 169.

Dent, C. E. & Harris, H. (1951). *Ann. Eugen., Lond.*, **16**, 60.

Partridge, S. M. (1948). *Biochem. J.* **42**, 238.

Partridge, S. M. & Brimley, R. C. (1951). *Biochem. J.* **48**, 313.

Westall, R. G. (1952). *Biochem. J.* **52**, 638.

A Chemical Examination of Connective Tissue in Rheumatic Fever

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A characteristic feature of subcutaneous nodules in rheumatic fever and also in rheumatoid arthritis is the central core of eosinophil fibrillar material, usually termed 'fibrinoid' from its histological resemblance to fibrin. However, there is much conflict of opinion regarding the presence of fibrin in such lesions, and these opinions are based mainly on histological grounds (cf. Glynn & Loewi, 1952), and few chemical or physical studies have been made on such tissue. Much stronger evidence for the presence of degraded collagen in rheumatoid arthritis is afforded by electron-micrograph and X-ray studies of Kellgren, Ball, Astbury, Reed & Beighton (1951), and chemical analyses of rheumatoid nodules have shown that the collagen present has its normal content of hydroxyproline (Bien & Ziff, 1951). The purpose of the present investigation was to examine chemically rheumatic fever nodules in order to ascertain what difference, if any, existed between them and normal connective tissue. A preliminary report of this work has been given by Consden, Glynn & Stanier (1952).

METHODS

The methods used were first chromatographic, which gave an overall picture of the amino-acid composition; and secondly, quantitative analysis of some of the amino acids and other constituents. As only a limited amount of tissue was available, and since this contained a mixture of different proteins, the analysis was restricted to those amino acids which would provide the most information. Therefore, those estimated were hydroxyproline, tyrosine and tryptophan. Since collagen is rich in hydroxyproline, and elastin is the only other animal protein which contains it in significant quantities, determination of this amino acid, after suitable fractionating procedures, provides an estimate of these two proteins, as has been shown by Neuman & Logan (1950*b*). Conversely, tryptophan, which is absent from collagen and allied proteins, is present in most other proteins, and estimation of this amino acid will, therefore, give a measure of protein constituents other than collagen and elastin. Tyrosine occupies an intermediate position, being present in only small amounts in collagen and elastin, but in much higher amounts in other proteins, especially in those of the blood. In addition to these amino acids, it was desirable to examine the carbohydrate constituents, since it is well established that polysaccharides occur in connective tissue.

Since nodules appear to contain a variable amount of unaltered collagen (Glynn & Loewi, 1952), it was desirable to separate this from non-collagenous material and to carry out analyses on both fractions. Collagen may be extracted by various methods, but it seemed preferable to use a mild method such as autoclaving with water at moderate pressure. The residue of connective tissue after autoclaving is usually regarded as being composed chiefly of elastin, though elastin from different sources does not always appear to be identical as regards amino-acid composition (Bowes & Kenten, 1949; Hall, 1951). Although collagen can be completely extracted by this method and elastin appears to be resistant, other insoluble proteins occupy an intermediate position. Nevertheless, this procedure affords a rough fractionation into a soluble collagen-rich and an insoluble non-collagen fraction. Both were analysed. Non-rheumatic subcutaneous tissue, from an area similar to that in which the nodules occurred, was also fractionated and analysed in order to provide a basis of comparison for the rheumatic tissue.

EXPERIMENTAL

Collection and pretreatment of tissues. Freshly excised, subcutaneous nodules from the elbow region were obtained from twelve rheumatic-fever patients of both sexes, whose ages ranged from 8 to 30 yr. Under the dissecting microscope, the nodules were freed as far as possible from adjacent, apparently normal, connective tissue. Part was retained for histological examination and the remainder was washed thoroughly with saline, then with water to remove salts and soluble proteins. After drying *in vacuo*, or alternatively, after dehydration by washing with successive portions of absolute ethanol, the tissue was extracted with benzene followed by light petroleum (b.p. 100–120°) to remove fatty material. The dried nodules thus obtained were small, ranging in weight from 1 to 15 mg. and were hygroscopic. A larger quantity of nodules was obtained *post mortem* from a female child aged 8 yr. who had died from congestive cardiac failure during rheumatic fever. After the treatments described above the specimens were pooled for quantitative analyses; they weighed 135 mg.

Non-rheumatic tissue was obtained *post mortem*. After careful removal of any epidermis, the tissue was treated as described above. Two samples were obtained, one from a male aged 38 yr. who had died from a duodenal fistula, and the other from a male aged 9 yr. who had died from thrombosis of the sagittal sinus. The air-dry weights were 123 and 360 mg. respectively. Both these tissues did not differ, histologically, from normal. Healthy tendo Achillis was collected *post mortem* from a male aged 62 yr., cut into small pieces, and treated as described above. This served as a normal collagen control.

Histological examination. For this purpose material was fixed in 5% formol-saline, embedded in paraffin and sectioned at 5μ. Sections were stained by a variety of techniques including haematoxylin and eosin, and Hotchkiss's periodic acid-Schiff reagent method for α-glycols. No nodule was used for chemical study unless typical 'fibrinoid' material was present on histological examination (Glynn & Loewi, 1952). The criteria for its recognition were intense eosinophilic amorphous and fibrillar material in the connective tissue giving a strongly positive reaction with the periodic acid-Schiff reagent and the presence of argyrophilic fibres in this material in sections stained by the silver impregnation method of Gomöri (1937).

Extraction of collagen. Tissues were autoclaved twice with water at 120° for 3 hr. in a sealed tube as described by Neuman & Logan (1950*b*). Extracts and washings were concentrated *in vacuo* to small bulk and finally dried *in vacuo* over H_2SO_4. Original tissues, extracts and residues were weighed after drying to constant weight over H_2SO_4 in a vacuum desiccator. The tendon was completely extractable by autoclaving.

Chromatography. Samples were hydrolysed for 17 hr. at 100–110° with 6N-HCl, repeatedly evaporated *in vacuo* to remove excess acid, and finally made up to a suitable volume with water. Two-dimensional paper chromatography (Consden, Gordon & Martin, 1944), was carried out on the hydrolysates using an amount of solution equivalent to about 1 mg. of protein. In earlier experiments, the solvents were collidine for the first direction, and phenol:coal gas: NH_3 for the second, but for most of the chromatograms the phenol was replaced by water-saturated *m*-cresol containing 8-hydroxyquinoline (0·1%). With the *m*-cresol system a longer period of development is required (40 hr.) than for phenol (17 hr.). Except for aspartic and glutamic acids, which are barely separated, the *m*-cresol system gives a more satisfactory separation of the amino acids. The faster moving amino acids are more satisfactorily resolved, e.g. methionine is separated from the leucines, and arginine and lysine are better separated from each other. Histidine is clearly visible, and cystine, even if present in very small amounts, is usually distinctly visible. In some cases, the presence of cystine was confirmed by oxidizing a portion of the hydrolysate with bromine water in order to convert the cystine to cysteic acid (Consden & Gordon, 1950), followed by one-dimensional chromatography in phenol:NH_3.

Examination of carbohydrates. The tissue was hydrolysed with N-H_2SO_4 at 100–110° for 3 hr., neutralized with $Ba(OH)_2$ and after separation from $BaSO_4$, concentrated so that 10μl. was equivalent to approx. 1 mg. of original substance. Sugars were examined by ionophoresis in

borate buffer at pH 8·6 according to the method of Consden & Stanier (1952*a*), in the apparatus described by Consden & Stanier (1952*b*), using 10–20 μl. of hydrolysate. Further separation of sugars was carried out by subsequent chromatography at right angles to the direction of ionophoresis, using the *n*-butanol:acetic acid system of Partridge (1948), except that the developing solvent was only about 80% saturated with water in order to overcome the effect of the salt on the paper. For ionophoresis alone, Whatman no. 1 paper was used; the two-dimensional separations were carried out on Whatman no. 42.

Test for hexosamine. The above hydrolysates were used for this procedure. 150 μl., equivalent to about 15 mg. protein, was applied along the starting line on a strip of Whatman no. 1 paper in the ionophoresis apparatus. Below this, and near one edge, was placed a control solution consisting of 5 μl. hydrolysate to which was added about 40 μg. glucosamine. The electrolyte solution consisted of 200 parts 0·67 M-acetic acid and 36 parts 0·67 M-$Ba(OH)_2$, its pH being 3·5. After 2 hr. at 10 v/cm., the paper was removed and dried. A strip containing the control was cut off and coloured with ninhydrin. The hexosamine spot occurred near the spots of lysine and arginine which had moved towards the cathode, whereas most of the ninhydrin-reacting constituents remained near the origin. By reference to the control strip, that portion of the remainder of the sheet containing the basic constituents was cut out and eluted with water. The eluate (1–2 ml.) was made acid to thymol blue with H_2SO_4, the precipitated $BaSO_4$ removed and acetic acid removed from the solution by repeated evaporation *in vacuo* with water. Excess H_2SO_4 was then removed by addition of $Ba(OH)_2$ to pH 5–6, the precipitate separated and the solution evaporated *in vacuo* to dryness. The solid was then dissolved in a small volume of water and transferred to a sheet of Whatman no. 4 paper for chromatography in *m*-cresol:hydroxyquinoline:NH_3 for 17 hr. Side by side with the test solution were placed the following control solutions: (*a*) glucosamine, (*b*) galactosamine, (*c*) a mixture of these two, and (*d*) an amino-acid mixture. Two chromatograms of controls and test solution were developed on the same sheet. After development and removal of solvent, the sheet was divided into two, so that each portion contained test and control solutions. One was treated with ninhydrin and the other with aniline hydrogen phthalate (Partridge, 1949). In this solvent system glucosamine is just separated from galactosamine, the former travelling at the rate of alanine and the latter at the rate of tyrosine. Both amino sugars with ninhydrin give grey and reddish-grey spots after a short heating period which gradually change to purple, galactosamine changing more slowly than glucosamine. In the test solution, strong spots of lysine and arginine occurred, but these were well separated from the hexosamine. However, relatively faint ninhydrin-reacting constituents occurred near the hexosamine positions, and were probably due to peptides. Aniline hydrogen phthalate was used as an additional test for the amino sugars, since amino acids and peptides do not react with this reagent, whereas the hexosamines behave similarly to the hexoses, both as regards the visible colour which is produced, and the fluorescence in ultraviolet light.

Quantitative analyses. Hydroxyproline was determined in the 6 N-HCl hydrolysates by the method of Neuman & Logan (1950*a*). It was found that rather larger amounts of hydroxyproline were necessary for satisfactory colour development than suggested by these authors, a satisfactory range being 0·025–0·075 mg. Tryptophan was determined by the method of Spies & Chambers (1948). For this analysis, samples were hydrolysed with 5 N-NaOH at 100° for 17 hr. Tyrosine was estimated after hydrolysis *in vacuo* with 6 N-HCl at 100–110° for 17 hr. by the method of Folin & Ciocalteu (1927). Ultraviolet spectrophotometric measurements of tyrosine and tryptophan (cf. Goodwin & Morton, 1946; Holiday, 1936) in the alkaline and acid hydrolysates were also undertaken and, although found to be not so consistent as the Folin-Ciocalteu method, there was fairly good agreement between the mean values determined by both methods. Nitrogen in samples of the hydrolysates was estimated by the micro-Kjeldahl method. Reducing sugars (as glucose) were estimated by the copper reduction method of Shaffer & Somogyi (1933). For this analysis the samples were hydrolysed with N-HCl at 100–110° for 3 hr. Hexosamine was estimated in the 6 N-HCl hydrolysates by the method of Elson & Morgan (1933), there being insufficient material for the hydrolysis to be carried out under the milder conditions, which are usually employed for hexosamine determination. Prolonged hydrolysis with strong acid is known to destroy a little of the hexosamine (Johnston, Ogston & Stanier, 1951); on the other hand, in the presence of much protein, hexosamine figures may be erroneously high (Johns & Marrack, 1952), which we have confirmed in recovery experiments, with gelatin containing added amounts of glucosamine (1–2%). The 'protein' effect probably more than counterbalances the hydrolysis loss, and our hexosamine figures may be 5–10% too high.

RESULTS

Autoclaving. The twelve individual nodules left residues varying between 25 and 40% of the original weight. The pooled nodules from one case left 30% residue (Table 1). The residue of each of the two samples of non-rheumatic connective tissue left residues of about 10% (Table 1).

Chromatograms. All nodule extracts gave, after hydrolysis, chromatograms similar to those of hydrolysed extracts of non-rheumatic tissue. These resembled chromatograms of hydrolysed tendon and gelatin, except that somewhat more tyrosine was present in the tissue extracts. The chromatograms of the extracts therefore resembled that of collagen (Bowes & Kenten, 1949), which is characterized by the presence of hydroxylysine and of very strong glycine, hydroxyproline and proline spots. All nodule-residue chromatograms resembled each other but differed markedly from the non-rheumatic residue chromatograms. The latter were characteristic of elastin, e.g. from cattle ligamentum nuchae (cf. Bowes & Kenten, 1949) in which the strongest spots are those of glycine, alanine, valine and the leucines; hydroxyproline, aspartic and glutamic acids, the hydroxyamino acids and the basic amino acids are relatively weak. The nodule residues, however, gave chromatograms suggesting the presence of protein additional to elastin, since those amino acids which were weak in elastin were distinctly stronger and cystine, absent from elastin and non-rheumatic residues, was

Table 1. *Analysis of subcutaneous elbow tissue*

	Amount after autoclaving (%)	Chromatogram type	Hydroxyproline (N as % total N)	Tyrosine (N as % total N)	Tryptophan (N as % total N)	Reducing sugar (as glucose, g./100 g. protein)	Hexosamine (g./100 g. protein)
Rheumatic fever, female, aged 8 yr.:							
Residue	30	Fibrin-elastin	0·7	3·4	0·8	3·6	0·9
Extract	70	Collagen	7·6	1·6	Trace	3·1	0·9
Non-rheumatic, male, aged 30 yr.:							
Residue	10	Elastin	1·1	2·5	—	—	—
Extract	90	Collagen	8·2	0·7	Trace	1·4	0·5
Non-rheumatic, male, aged 9 yr.:							
Residue	9	Elastin	0·9	3·5	—	2·3	—
Extract	91	Collagen	7·7	0·9	—	2·2	0·5
Comparative data:							
Gelatin*	—	—	8·6	0·4	0	0·2†	Trace†
Collagen*	—	—	8·0	0·4–0·6	0	0·4‡	0·2;‡ 0·3§
Elastin*	—	—	1·0–1·3	0·6–0·7	0	—	—
Human fibrin‖	—	—	0	2·7	2·7	4·5	1·1

* Data for amino-acid composition of bovine, bovine collagen and elastin (animal and human) from Tristram (1949), Bowes & Kenten (1949), Neuman & Logan (1950*b*) and Neuman (1949).
† Commercial bovine gelatin (present paper).
‡ Human tendo Achillis (present paper).
§ From Bowes (1951).
‖ Amino-acid analyses, present paper; reducing sugar and hexosamine data from Consden (1953*a*).

usually present in small amount in the nodule residues. Chromatograms of the latter were similar to that of a mixture of fibrin and elastin and are therefore referred to as 'fibrin-elastin' (see Table 1), though it is not necessarily implied that only these two proteins are present.

Qualitative examination of carbohydrates. Nodules from several individuals were pooled, autoclaved and the fractions analysed for carbohydrates. The hexosamine in the extract was glucosamine; no galactosamine was found. This was true also of non-rheumatic tissue extract. The sugars present in hydrolysates of rheumatic and non-rheumatic residue and extract were found, by ionophoresis, to be mannose, galactose and glucose in addition to hexosamine; uronic acid was absent. In all the hydrolysates galactose was present in distinctly higher amount than the other two hexoses and glucose was relatively weaker than mannose. A two-dimensional separation (ionophoresis followed by chromatography) of a hydrolysate (1 mg. tissue) with added galactose and mannose (10 μg. each) was no different from that of the hydrolysate alone, except that the strengths of these two sugar spots were enhanced in the former case. A two-dimensional separation which is typical of all the hydrolysates is illustrated in Fig. 1.

Quantitative data. Quantitative data for the rheumatic-fever tissue from one individual and for the two non-rheumatic tissues are given in Table 1, together with comparative data for other proteins.

DISCUSSION

Chromatography and tryptophan and hydroxyproline figures suggest that both rheumatic and non-rheumatic extracts are similar to each other

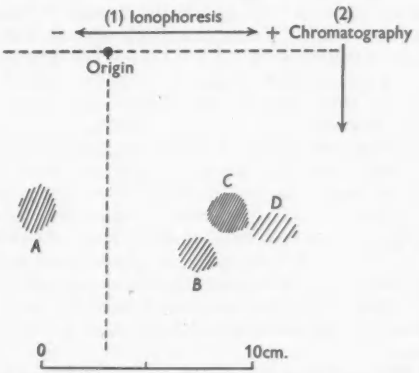

Fig. 1. Two-dimensional separation of tissue hydrolysate on Whatman no. 42 paper. Ionophoresis in borate, pH 8·6 (3 hr. at 10 v/cm.) and chromatography in *n*-butanol:acetic acid (41 hr.) in directions shown. Aniline hydrogen phthalate spots: *A*, glucosamine; *B*, mannose (moderate-weak); *C*, galactose (strong); *D*, glucose (weak).

and are mainly collagen. In view of possible variations between individuals, the lower hydroxyproline of the rheumatic residue compared with that of non-rheumatic residue may not be significant.

However, if the hydroxyproline is due to elastin, then this protein amounts to 50–70 % of the nodule residue. Its tryptophan content would correspond to about 30 % fibrin, but a mixture of these two proteins in the above proportions would give a tyrosine figure of about 1 %, which is much lower than that actually found. Further, it was found that over 50 % of fibrin was extractable by autoclaving a preparation from a fibrin clot; therefore, if the nodule residue contained 30 % fibrin, an equal amount should have been present in the nodule extract. This would have made the tryptophan figure at least 0·3 % in the extract, whereas it was in fact much smaller. It would therefore appear that fibrin, if present in nodules, does not occur to the extent of more than a few per cent. The high tyrosine content of all the residues suggests the presence of a tyrosine-rich protein or polypeptide. However, neither of the methods used for tyrosine estimation is specific for this amino acid but are also methods for phenolic compounds in general and the possibility that the tyrosine figures may include values for non-tyrosine phenolic compounds cannot be ignored. Nevertheless, chromatograms of all hydrolysed residues, both of rheumatic and non-rheumatic, suggest that there is indeed more tyrosine than in tendon, gelatin and elastin. Although the tyrosine content of non-rheumatic residue is comparable in amount with that of rheumatic residue, the absolute quantity in the nodules is much higher than in non-rheumatic tissue, since the residue of the former is much greater. Similar conclusions apply to the carbohydrate constituents. In both rheumatic and non-rheumatic tissues, the sugars released on hydrolysis are the same, and in both cases they appear to occur in similar relative proportions to each other. This suggests that the polysaccharide components normally present in subcutaneous tissue occur also in rheumatic lesions. There is little reason for supposing that the nodules contain substances which are not normally present, but the results suggest that the non-collagen protein and polysaccharide occur locally in higher amounts in the nodules. This may be due either to increased deposition of these substances or to destruction and removal of collagen.

The analytical results of non-rheumatic tissue itself call for some comment. The existence of acidic mucopolysaccharides in the interfibrillary substance of the mesenchyme has long been postulated, and both hyaluronic acid and chondroitin sulphate have been isolated from animal skin by Meyer & Chaffee, 1941 (cf. Rienits, 1953), and from human skin by Pearce & Watson (1949). The absence of uronic acid and galactosamine in the mild hydrolysates of the tissue, whilst not evidence for the absence of hyaluronic acid and chondroitin sulphate, in view of the small quantities used for analysis, does indicate that, if present in subcutaneous tissue, the amounts of these polysaccharides are small compared with that of other uronic-acid-free polysaccharides. Further, if hyaluronic acid were present in sufficient amount, mild hydrolysis would have also given hyalobiuronic acid (Rapport, Weissmann, Linker & Meyer, 1951), which has always been observed in hydrolysates of hyaluronic acid preparations (Consden, 1953*b*). The presence of galactose in the hydrolysates of tissue, apparently as a major sugar constituent, suggests the presence of a galactose-rich polysaccharide. Some of this material, particularly in the residues, would appear to form part of the interfibrillary ground substance, especially since Little & Kramer (1952) have shown that this is resistant to boiling water. Few chemical studies have been carried out on this material, although it is generally accepted that it is mucoprotein in nature. Our high tyrosine values in the residue are in line with the work of Lisco (1949), who found evidence for the presence of protein, rich in aromatic amino acid, in beef and rat-tail tendons, but it is evident that further chemical work on the non-collagen, non-elastin constituents of normal subcutaneous tissue itself is necessary before one can obtain a clearer picture of the changes in pathological tissue.

SUMMARY

1. Subcutaneous elbow nodules from rheumatic-fever patients have been fractionated by autoclaving into collagen-rich extracts and non-collagen residues. Qualitative and quantitative analysis of the hydrolysed fractions were carried out and compared with analyses of non-rheumatic subcutaneous elbow tissue treated similarly.

2. In addition to collagen and elastin, the nodules contained a tyrosine-rich substance and polysaccharide. These appear to be present in non-rheumatic tissue, but in much smaller amount. The presence of large quantities of fibrin in nodules is regarded as improbable.

3. The predominant polysaccharide constituents of both non-rheumatic and rheumatic subcutaneous tissues are rich in galactose and glucosamine.

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REFERENCES

Bien, E. G. & Ziff, M. (1951). *Proc. Soc. exp. Biol., N.Y.*, **78**, 327.
Bowes, J. H. (1951). *Research, Lond.*, **4**, 155.
Bowes, J. H. & Kenten, R. H. (1949). *Biochem. J.* **45**, 281.
Consden, R. (1953*a*). *Disc. Faraday Soc.* **13**, 282.
Consden, R. (1953*b*). *Disc. Faraday Soc.* **13**, 286.
Consden, R., Glynn, L. E. & Stanier, W. M. (1952). *Biochem. J.* **50**, xix.
Consden, R. & Gordon, A. H. (1950). *Biochem. J.* **46**, 8.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
Consden, R. & Stanier, W. M. (1952*a*). *Nature, Lond.*, **169**, 783.
Consden, R. & Stanier, W. M. (1952*b*). *Nature, Lond.*, **170**, 1069.
Elson, L. E. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
Folin, O. & Ciocalteu, V. (1927). *J. biol. Chem.* **73**, 627.
Glynn, L. E. & Loewi, G. (1952). *J. Path. Bact.* **64**, 329.
Gomöri, G. (1937). *Amer. J. Path.* **13**, 993.
Goodwin, T. W. & Morton, R. A. (1946). *Biochem. J.* **40**, 628.
Hall, D. A. (1951). *Nature, Lond.*, **168**, 513.
Holiday, E. R. (1936). *Biochem. J.* **30**, 1795.
Johns, R. G. S. & Marrack, J. R. (1952). *Biochem. J.* **50**, xvii.
Johnston, J. P., Ogston, A. G. & Stanier, J. E. (1951). *Analyst*, **76**, 899.
Kellgren, J. H., Ball, J., Astbury, W. T., Reed, R. & Beighton, E. (1951). *Nature, Lond.*, **168**, 493.
Lisco, H. (1949). *Arch. Biochem.* **22**, 406.
Little, K. & Kramer, H. (1952). *Nature, Lond.*, **170**, 499.
Meyer, K. & Chaffee, E. (1941). *J. biol. Chem.* **138**, 491.
Neuman, R. E. (1949). *Arch. Biochem.* **24**, 289.
Neuman, R. E. & Logan, M. A. (1950*a*). *J. biol. Chem.* **184**, 299.
Neuman, R. E. & Logan, M. A. (1950*b*). *J. biol. Chem.* **186**, 549.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
Pearce, R. H. & Watson, E. M. (1949). *Canad. J. Res.* **27** E, 43.
Rapport, M. M., Weissmann, B., Linker, A. & Meyer, K. (1951). *Nature, Lond.*, **168**, 996.
Rienits, K. G. (1953). *Biochem. J.* **53**, 79.
Shaffer, P. A. & Somogyi, M. (1933). *J. biol. Chem.* **100**, 695.
Spies, J. R. & Chambers, D. C. (1948). *Analyt. Chem.* **20**, 30.
Tristram, G. R. (1949). *Advanc. Protein Chem.* **5**, 84.

Studies on Sulphatases

3. THE ARYLSULPHATASE AND β-GLUCURONIDASE OF MARINE MOLLUSCS

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Arylsulphatase was first demonstrated in molluscs by Derrien (1911) who found that extracts of a large marine gastropod, *Murex trunculus*, could hydrolyse indoxyl sulphate. Soda & Egami (1933) and Soda (1936) studied the distribution of the enzyme in a large number of tropical marine molluscs common to Japanese waters. Considerable activity was observed amongst members of the Gastropoda, Pelecypoda, Cephalopoda and Amphineura. It was also found in some Arthropoda. In all cases the enzyme activity was mainly concentrated in the digestive glands. Information regarding the properties of the mollusc enzyme is scanty. Soda (1936) recorded an optimum pH of 7 for the arylsulphatase of *Charonia lampas* (*Tritonium nodiferum*) when potassium phenylsulphate was the substrate, although Soda & Egami (1941) used pH 5·2 for enzyme assay. The substrate concentration used by these workers (0·05 M) was apparently arbitrary and not necessarily optimal. The high arylsulphatase activity of extracts of the digestive glands of *Haliotis gigantea* (Soda & Koyama, 1935) was confirmed by Morimoto (1937) using potassium *p*-nitrophenylsulphate as substrate under arbitrary experimental conditions. Tanaka (1938) found that the maximum hydrolysis of potassium *p*-nitrophenylsulphate by aqueous extracts of digestive glands of various marine organisms occurred at pH 5·0–5·2 when the substrate concentration was 0·0005 M. The optimum substrate concentration was not recorded.

During preliminary studies (Dodgson, Lewis & Spencer, 1952) the digestive organs of many marine molluscs common to British waters were found to be rich in arylsulphatase and promising as starting material for the preparation of enzyme concentrates. It was noted that the organisms also possessed marked β-glucuronidase activity. Nagaoka (1951) used the β-glucuronidase of certain terrestrial snails, but there appears to be no previous record of the occurrence of this enzyme in marine molluscs. In view of the desirability of

removing β-glucuronidase from preparations of arylsulphatase, and because of the comparative dearth of information regarding the optimum conditions for the activity of the two enzymes in invertebrate organisms, it was decided to investigate these conditions. The large periwinkle (*Littorina littorea*) and the limpet (*Patella vulgata*) were selected for this study since they were both readily accessible locally and rich sources of both enzymes. Using the optimum conditions established for these two organisms, the arylsulphatase and β-glucuronidase activities of a number of other marine molluscs have been examined in order to throw further light on the problem of the physiological significance of the two enzymes. A preliminary account of this work has already been published (Dodgson *et al.* 1952).

MATERIALS AND METHODS

Supplies of *Littorina* and *Patella* were obtained locally from the Bristol Channel area; other organisms were obtained from the Marine Biological Station, Plymouth. In the laboratory they were kept alive in aerated sea-water or, for shorter periods, in the ice-chest.

Substrates. Potassium *p*-acetylphenylsulphate, free from *p*-hydroxyacetophenone, was prepared as previously described (Dodgson & Spencer, 1953). *p*-Chlorophenylglucuronide monohydrate was prepared biosynthetically (Spencer & Williams, 1951).

Tissue suspensions. These were prepared using a glass homogenizer of the Potter-Elvehjem type (see Potter & Elvehjem, 1936; Brown, Dodgson, Sherwood & Spencer, 1952).

Determination of arylsulphatase activity. The spectrophotometric method of Dodgson, Spencer & Thomas (1953) using potassium *p*-acetylphenylsulphate as substrate was modified by omission of ethanol. When ethanol was used as a protein precipitant with mollusc digests the addition of NaOH gave cloudy solutions which could not be clarified by centrifugation. Complete removal of protein from the digests was not necessary since protein solutions do not absorb strongly at 323 mμ. Enzyme action could be stopped by 0·2 N-NaOH and, at the concentrations of tissues used in these experiments (usually 0·1% wet wt./vol.), solutions sufficiently clear for spectrophotometric measurement could be obtained by centrifuging at 2000 *g* for 15 min. The procedure was identical with that of Dodgson *et al.* (1953) except that 4·8 ml. of 0·2 N-NaOH were used instead of 4·8 ml. of ethanol. After centrifuging, the absorptions of the solutions in 1 cm. cells were measured on a Hilger Uvispek spectrophotometer at 323 mμ. without further treatment. Under these conditions the expression $(E_t - E_c) \times 40{\cdot}84$ (where E_t and E_c are the observed extinctions of the test and control solutions respectively) gives the amount of the liberated *p*-hydroxyacetophenone in μg.

Determination of β-glucuronidase activity. The method was based on that of Spencer & Williams (1951) using *p*-chlorophenylglucuronide monohydrate as substrate. Ethanol was omitted for the reason given in the preceding section. At the tissue concentrations used for the measurement of β-glucuronidase activity (approx. 1% wet wt./vol.), neither 0·2 N-NaOH nor KOH could be substituted for ethanol since the cloudy solutions obtained could not be clarified by centrifugation but 0·35 N-$Ba(OH)_2$ gave clear solutions providing precautions were taken to prevent the formation of $BaCO_3$ during the centrifuging and subsequent spectroscopic procedure. The amount of $Ba(OH)_2$ was sufficient to convert the *p*-chlorophenol to the anionic form. Any traces of protein which remained in solution did not greatly interfere with the determinations since protein does not absorb very strongly at 298 mμ.

To each of four 15 ml. centrifuge tubes was added 0·4 ml. of tissue suspension in 0·5 M-acetate buffer of the required pH. All suspensions were kept at 0° before incubation (see p. 256). The four tubes were stoppered and placed at 1 min. intervals in a water bath at 37·5°. After allowing 4 min. for each suspension to attain the temperature of the bath, 0·4 ml. of a suitably buffered solution of *p*-chlorophenylglucuronide monohydrate was added to the first two tubes (tests). After incubating each tube for exactly 1 hr., 5·0 ml. of 0·35 N-$Ba(OH)_2$ were added to the first two tubes; 0·4 ml. substrate, followed immediately by 5·0 ml. of 0·35 N-$Ba(OH)_2$, was added to the second two tubes (controls). After clarifying the solutions by centrifugation, the extinctions of these solutions at 298 mμ. were measured on the spectrophotometer using 1 cm. cells. The amount of *p*-chlorophenol (μg.) liberated by the enzyme is given by the expression

$$(E_t - E_c) \times 286{\cdot}6,$$

where E_t and E_c are the observed extinctions of the test and control solutions respectively.

RESULTS

Arylsulphatase

The characteristics of arylsulphatase. Suspensions of both *Littorina* and *Patella* viscera exhibited maximum enzyme activity at pH 5·5 in 0·5 M-acetate buffer (Fig. 1). At this pH the optimum concentration of substrate was 0·065 M for Patella and 0·04 M for *Littorina* (Fig. 2). Under these optimum conditions the release of *p*-hydroxyacetophenone by both enzyme preparations was not rectilinearly related to the time of incubation (Fig. 3). The decrease in activity with time is apparently not due to the formation of inhibitory end-products of the reaction since for both preparations there was a rectilinear relationship between the extent of hydrolysis of *p*-acetylphenylsulphate and the concentration of enzyme.

Effect of incubation in the absence of substrate. Dodgson *et al.* (1953) have shown that preliminary incubation of rat-liver suspensions in the absence of substrate results in a loss of arylsulphatase activity. *Littorina* and *Patella* preparations also lost enzyme activity when incubated before addition of substrate. In a typical experiment the activities of *Littorina* and *Patella* suspensions had declined to 79·5 and 70·5% respectively of the initial value after incubation for 1 hr. at 37·5°. This inactivation was still appreciable at room temperature, but there was no loss of enzyme activity in enzyme preparations

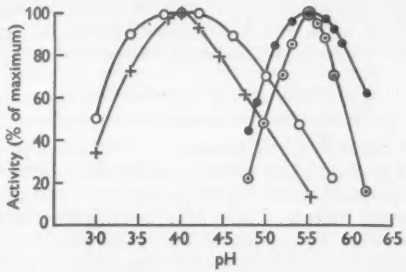

Fig. 1. pH activity curves for the arylsulphatases and β-glucuronidases of visceral-hump suspensions of *Patella* and *Littorina*. For arylsulphatase the respective concentrations of potassium *p*-acetylphenylsulphate were 0·065 M and 0·04 M in 0·5 M-acetate buffer. For β-glucuronidase the concentration of *p*-chlorophenylglucuronide was 0·045 M in 0·5 M-acetate buffer. Incubations were for 1 hr. at 37·5°. ●—●, *Patella* arylsulphatase; ⊙—⊙, *Littorina* arylsulphatase; ○—○, *Patella* β-glucuronidase; ×—×, *Littorina* β-glucuronidase.

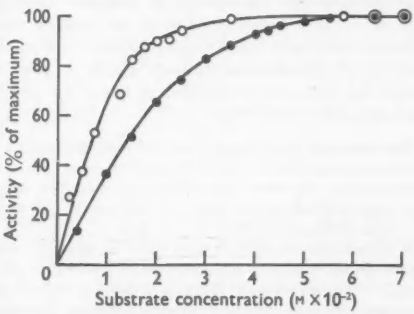

Fig. 2. Substrate concentration-activity curves for the arylsulphatases of *Patella* and *Littorina* acting on potassium *p*-acetylphenylsulphate in 0·5 M-acetate buffer, pH 5·5, during 1 hr. at 37·5°. ●—●, *Patella*; ○—○, *Littorina*.

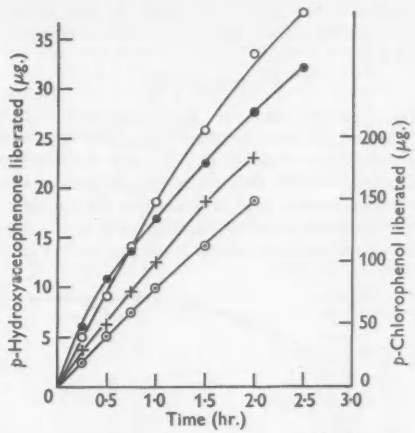

Fig. 3. Time-activity curves for the arylsulphatases and β-glucuronidases of *Patella* and *Littorina* at 37·5° using optimum concentrations of potassium *p*-acetylphenylsulphate in 0·5 M-acetate buffer, pH 5·5, and *p*-chlorophenylglucuronide in 0·5 M-acetate buffer, pH 4·0. ●—●, *Patella* arylsulphatase; ○—○, *Littorina* arylsulphatase; ⊙—⊙, *Patella* β-glucuronidase; ×—×, *Littorina* β-glucuronidase.

maintained at 0° for several hours. For this reason all enzyme preparations were kept at 0° before assay.

The fractionation of arylsulphatase in buffered suspensions. Dodgson *et al.* (1953) noted that whole suspensions of rat liver must be used in the assay of rat-liver arylsulphatase, since only about 60–80% of the total enzyme activity is present in supernatant solutions after the suspensions have been centrifuged at 2000 *g* for 20 min. This also applies to mollusc preparations (Table 1). As with rat arylsulphatase the total activity of mollusc suspensions is not obtained when the supernatant

Table 1. *The fractionation of mollusc arylsulphatase and β-glucuronidase*

(Suspensions were centrifuged at 2000 *g* for 15 min. at 0°, and the supernatant solutions and debris assayed separately using the optimum conditions for enzyme activity. The activities of the whole suspensions were determined simultaneously. Values are expressed as percentage of activity of whole suspension.)

Organism	Suspension medium	Activity: Supernatant	Activity: Residue	Loss (%)
	Arylsulphatase			
Littorina littorea	Water	80·5	7·4	12·1
	0·5 M-acetate buffer, pH 5·5	79·0	6·8	14·2
Patella vulgata	Water	87·6	3·5	8·9
	0·5 M-acetate buffer, pH 5·5	71·5	16·8	11·7
	β-Glucuronidase			
Littorina littorea	Water	73·6	31·2	—
	0·5 M-acetate buffer, pH 4·0	66·7	31·5	1·8
Patella vulgata	Water	92·0	9·5	—
	0·5 M-acetate buffer, pH 4·0	78·3	21·5	0·2

solution and the debris are separated and assayed independently. This point is being investigated further.

β-Glucuronidase

The characteristics of β-glucuronidase. Both *Patella* and *Littorina* preparations showed greatest β-glucuronidase activity at pH 4·0 in 0·5 M-acetate buffer (Fig. 1). At this optimum pH both enzyme preparations were most active when the concentration of substrate was 0·045 M. Greater concentrations of substrate showed no inhibitory effect (Fig. 4).

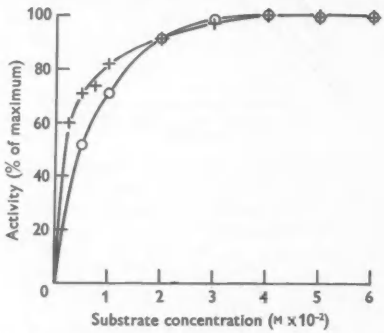

Fig. 4. Substrate concentration-activity curves for the β-glucuronidases of *Patella* and *Littorina* acting on *p*-chlorophenylglucuronide monohydrate in 0·5 M-acetate buffer at pH 4·0 at 37·5° during 1 hr. ×—×, *Patella*; ○—○, *Littorina*.

There was not a straight-line relationship between the time of incubation and β-glucuronidase activity of either *Patella* or *Littorina* preparations, using the optimum conditions of pH and substrate concentration, although over the period of the first hour the curves were very nearly rectilinear (Fig. 3). A graph relating the enzyme concentration with the degree of hydrolysis of *p*-chlorophenylglucuronide gave the expected rectilinear curves.

Effect of incubation in the absence of substrate. Incubation of the enzyme preparations in the absence of substrate resulted in a small but steady loss of enzyme activity. In typical experiments the activity of *Littorina* and *Patella* preparations after incubation for 1 hr. had declined to 84 and 97 % respectively of the initial activities. It is thus necessary to keep all enzyme preparations at 0° before assay.

Fractionation of the enzyme in buffered suspensions. The β-glucuronidase activity of both organisms is distributed between supernatant solution and debris when suspensions are centrifuged at 2000 *g* for 15 min. (Table 1). It is therefore necessary to use whole suspensions in order to realize maximum enzyme activity. In contrast to arylsulphatase there appears to be no loss in β-glucuronidase activity when the supernatant solution and the debris are separated and assayed independently.

The distribution of arylsulphatase and β-glucuronidase in various marine molluscs

It has been shown that the optimum conditions for β-glucuronidase activity in *Littorina* and *Patella* are identical. These conditions have been used when assaying β-glucuronidase activities of other marine organisms. The optimum conditions for the arylsulphatase activity of the two organisms, however, are not identical, for although the pH optima are the same (pH 5·5) the optimum substrate concentration of *Patella* (0·065 M) is higher than that of *Littorina* (0·045 M). In neither case was there any inhibition by excess of substrate and for the assay of arylsulphatase activity of other molluscs the higher substrate concentration has been used. Since Dodgson *et al.* (1952) indicated that the arylsulphatase activity of marine molluscs can vary with their nutritional state, and Soda (1936) suggested possible seasonal variations of enzyme activity, organisms were assayed as soon as possible after collection and only those results obtained during the month of November are quoted. As far as possible within each species organisms of the same weight were selected.

The required organs were dissected out and washed in ice-cold distilled water for a few seconds in order to remove contaminating sea water. After being dried on filter paper, they were weighed and suspended in chilled distilled water for 2 min. using a chilled glass homogenizer. A portion of each suspension was diluted with distilled water for arylsulphatase assay to give a solution, 0·6 ml. of which would liberate between 15 and 30 μg. *p*-hydroxyacetophenone; the remainder was diluted to give a solution, 0·4 ml. of which would liberate between 100 and 200 μg. *p*-chlorophenol for β-glucuronidase assay. The appropriate amount of diluted suspension was then incubated for 1 hr. at 37·5° with an equal amount of the substrate in 1·0 M-acetate buffer of the required pH.

The results recorded in Table 2 show that all organisms tested were highly active with respect to both enzymes.

DISCUSSION

Although the present survey of the distribution of arylsulphatase and β-glucuronidase in the *Mollusca* is not comprehensive, there is no doubt that many of these organisms are particularly rich sources of the two enzymes. In order to make a balanced distribution survey several important factors would have to be considered. Thus during the present work it has been noted that the activities due to both enzymes increase considerably if the organisms are starved. This may be an apparent increase due to the elimination of material from the alimentary canal

Table 2. *Arylsulphatase and β-glucuronidase activities of various molluscs*

(Arylsulphatase activity was determined at pH 5·5 using 0·065 M-potassium *p*-acetylphenylsulphate in 0·5 M-acetate buffer. β-Glucuronidase activity was determined at pH 4·0 using 0·045 M-*p*-chlorophenylglucuronide monohydrate in 0·5 M-acetate buffer. Activities are expressed as μg. phenol liberated/g. wet tissue/hr. Ranges are given in parentheses.)

Organism	Organs taken	No. of expt.	β-Glucuronidase activity	Arylsulphatase activity
Calliostoma zizyphinum	Visceral hump	6	8 138 (2 950–15 480)	37 592 (27 900–45 600)
Nucella lapillus		5	9 084 (1 350–17 500)	13 450 (1 476–36 000)
Osilinus lineatus		5	53 180 (42 300–71 000)	284 200 (164 000–382 500)
Littorina littorea		5	19 780 (14 400–25 200)	54 140 (42 800–82 500)
Patella vulgata		5	44 925 (18 420–57 400)	98 500 (57 600–141 000)
Gibbula umbilicalis		5	43 608 (31 700–68 000)	127 360 (64 000–202 000)
G. cineraria	Whole organism	5	4 988 (2 448–7 616)	27 540 (12 400–38 600)
Buccinum undatum	Posterior digestive gland	3	8 950 (5 220–13 950)	66 633 (49 200–89 400)
	Anterior digestive gland	3	1 490 (910–2 070)	10 576 (3 850–21 300)
	Ovary	1	2 990	4 120
Scaphander lignarius	Visceral hump	3	2 336 (1 248–3 022)	12 970 (7 110–17 800)
Mytilus edulis	Digestive glands	5	13 912 (4 740–30 600)	49 900 (28 700–76 000)
Ostrea edulis	Digestive glands	4	10 078 (7 850–17 080)	12 935 (5 140–20 800)

during starvation, or a true increase. It would seem important to select organisms of similar age, sex and size. This is not easy for, although Moore (1936, 1937) has outlined methods for determining the sex and age of *Littorina*, these are not generally applicable to other organisms. Finally, it would be necessary to locate the enzymes more precisely within each species. The term 'visceral hump' is vague but it usually includes the digestive glands, the ovaries or testes, the alimentary canal and the kidney. In the larger organisms it is comparatively easy to recognize and dissect out the individual organs, but in the smaller organisms this is often impracticable. Moreover, although the work of Soda (1936) shows that the arylsulphatase activity of tropical molluscs is confined mainly to the digestive glands, this may not necessarily apply to the present study. Indeed it is possible that enzyme activity might be partially microbial in origin. Karunairatnam & Levvy (1951) have shown that the β-glucuronidase activity of sheep rumen is due to bacteria, and Marsh, Alexander & Levvy (1952) have suggested that the high β-glucuronidase activity found in the omasum, rumen, caecum and colon of many animals might well have a similar origin. Buehler, Katzman, Doisy & Doisy (1949) and Barber, Brooksbank & Haslewood (1948) have also revealed the potent β-glucuronidase activity of certain bacteria and Whitehead, Morrison & Young (1952) have found arylsulphatase activity in certain strains of salmonellae and mycobacteria. The possibility that the activity of β-glucuronidase and arylsulphatase in molluscs is due in part to bacteria or other parasites cannot be excluded, and is being studied further.

In a study of glucosulphatase and arylsulphatase in molluscs, Soda (1936) noted a correlation between the distribution of the two enzymes. The results of the present work suggest that the distribution of arylsulphatase and β-glucuronidase may be similar. Thus the activity of both enzymes appears to be very much greater in *Littorina*, *Patella*, *Osilinus* and *Gibbula umbilicalis* than in *Ostrea*, *Scaphander*, *Mytilus* and others. However, there is an insufficient number of experiments to justify such a conclusion.

Among the organisms studied enzyme activity cannot be correlated with habitat or feeding habits since some are relatively active carnivora living permanently under water (e.g. *Buccinum* and *Scaphander*) whereas others are relatively inactive herbivora periodically exposed to the atmosphere

Table 3. *The optimum* pH *and substrate concentrations for arylsulphatases of different origin, when potassium* p*-acetylphenylsulphate is the substrate*

Source	In 0·5 M-acetate buffer		In 0·2 M-phosphate buffer	
	Opt. pH	Opt. substrate concentration ($M \times 10^{-3}$)	Opt. pH	Opt. substrate concentration ($M \times 10^{-3}$)
Fungal				
Taka diastase (Parke, Davis and Co.) containing added lactose	6·2–6·4	4	7·6	3·5
Molluscan				
Patella vulgata	5·5	65	No activity	
Littorina littorea	5·5	40	No activity	
Mammalian				
Rat	7·2	7	8·2*	3·5*
Man	6·9	5	7·3	8

* Results from an acetone-dried preparation of rat liver.

by the retreating tide (*Patella*, *Littorina* and *Mytilus*). No clue is thus afforded as to the physiological significance of the two enzymes in these organisms. It is possible that β-glucuronidase may play a digestive role since many of the marine algae on which some of these organisms feed contain polysaccharide material which is rich in uronic acid residues. The function of arylsulphatase is even more obscure since the existence of naturally occurring substrates of the enzyme in the marine environment has not been established.

The high arylsulphatase and β-glucuronidase activities found in molluscs during this work probably do not represent the true picture of the activities of the enzymes in the intact organism in their marine environment. For instance the activity of the arylsulphatase of *Littorina* and *Patella* is inhibited to the extent of about 15 and 33% respectively in the presence of 50% sea water, although β-glucuronidase activity of the organisms is largely unaffected. Moreover, the activity of all the enzyme preparations is increased seven or eight times by raising the temperature of incubation from 10° to 37·5°. However, these facts do not lessen the possible value of molluscs as starting material for the preparation of highly active enzyme concentrates. Dodgson *et al.* (1952) have obtained mollusc arylsulphatase concentrates with activities many times greater than those from other sources, and it seems likely that highly active β-glucuronidase preparations could be prepared from the same material.

Table 3 shows the optimum pH values and optimum substrate concentrations for arylsulphatases of different origin which have been studied in this laboratory using potassium *p*-acetylphenylsulphate as substrate. The optimum conditions for the arylsulphatases of the rat, *Aspergillus oryzae*, and man are similar but very different from those for the enzyme from molluscs. This is true whether the buffer be acetate or phosphate. Unfortunately, mollusc β-glucuronidase is not comparable with β-glucuronidases from other sources which have been studied using substrates other than *p*-chlorophenylglucuronide. It is of interest, however, that there is as yet no evidence that the mollusc enzyme possesses more than one pH optimum. This is in contrast to the findings of Mills (1948), Mills & Paul (1949) and Spencer & Williams (1951) for ox-spleen β-glucuronidase, and Kerr, Campbell & Levvy (1949) for the mouse-liver enzyme.

SUMMARY

1. Using potassium *p*-acetylphenylsulphate and *p*-chlorophenylglucuronide monohydrate as substrates, the conditions necessary for the activity of the arylsulphatases and β-glucuronidases respectively of *Patella vulgata* and *Littorina littorea* have been studied.

2. Using the optimum conditions established for these organisms a short survey of the distribution of the two enzymes in other molluscs has been made.

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REFERENCES

Barber, M., Brooksbank, B. W. L. & Haslewood, G. A. D. (1948). *Nature, Lond.*, **30**, 701.
Brown, D. H., Dodgson, K. S., Sherwood, T. H. & Spencer, B. (1952). *Biochem. J.* **51**, xlvii.
Buehler, H. J., Katzmann, P. A., Doisy, P. P. & Doisy, E. A. (1949). *Proc. Soc. exp. Biol., N.Y.*, **72**, 297.
Derrien, M. (1911). *Bull. Soc. Chim. biol., Paris*, **9**, 110.
Dodgson, K. S., Lewis, J. I. M. & Spencer, B. (1952). *Biochem. J.* **51**, xlii.
Dodgson, K. S. & Spencer, B. (1953). *Biochem. J.* **53**, 444.
Dodgson, K. S., Spencer, B. & Thomas, J. (1953). *Biochem. J.* **53**, 452.
Karunairatnam, M. C. & Levvy, G. A. (1951). *Biochem. J.* **49**, 210.
Kerr, L. M. H., Campbell, J. G. & Levvy, G. A. (1949). *Biochem. J.* **44**, 487.
Marsh, C. A., Alexander, F. & Levvy, G. A. (1952). *Nature, Lond.*, **170**, 163.
Mills, G. T. (1948). *Biochem. J.* **43**, 125.
Mills, G. T. & Paul, J. (1949). *Biochem. J.* **44**, xxxiv.
Moore, D. M. (1936). *J. Mar. biol. Ass. U.K.* **20**, 72.
Moore, D. M. (1937). *J. Mar. biol. Ass. U.K.* **21**, 721.
Morimoto, K. (1937). *J. Biochem., Tokyo*, **26**, 259.
Nagaoka, T. (1951). *Tohoku J. exp. Med.* **53**, 29.
Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
Soda, T. (1936). *J. Fac. Sci. Tokyo Univ.* **3**, 150.
Soda, T. & Egami, F. (1933). *J. chem. Soc. Japan*, **54**, 1069.
Soda, T. & Egami, F. (1941). *J. chem. Soc. Japan*, **62**, 256.
Soda, T. & Koyama, S. (1935). *J. chem. Soc. Japan*, **56**, 1388.
Spencer, B. & Williams, R. T. (1951). *Biochem. J.* **48**, 537.
Tanaka, S. (1938). *J. Biochem., Tokyo*, **28**, 119.
Whitehead, J. E. M., Morrison, A. R. & Young, L. (1952). *Biochem. J.* **51**, 585.

The Effect of Cobalamin on the Quantitative Utilization of Serine, Glycine and Formate for the Synthesis of Choline and Methyl Groups of Methionine

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It was observed by Du Vigneaud, Chandler, Moyer & Keppel (1939) that rats generally lost weight when kept on a synthetic diet containing homocystine but deficient in methionine and choline. The favourable effect on growth of the addition of choline or betaine indicated the occurrence of transmethylation which was conclusively proved later by isotope experiments carried out in Du Vigneaud's laboratory. In these early experiments it was noticed that occasionally some animals on the deficient diets grew, but this was ascribed to the action of the intestinal flora. These observations were greatly extended by Bennett and co-workers (Bennett, Medes & Toennies, 1944; Bennett & Toennies, 1946) who also obtained evidence suggesting that factors present in liver extract are involved in the synthesis of labile methyl groups. That such a synthesis indeed occurred was shown with the aid of deuterium by Du Vigneaud, Simmonds, Chandler & Cohn (1945). However, the relatively small amount of deuterium in the choline methyl groups indicated that only about 8% of them was derived from the body water, the rest presumably originating from the methionine also present in the diet. This incorporation was again ascribed to micro-organisms.

More precise information about this synthesis of methyl groups in the rat was obtained by the use of carbon isotopes. Thus, one of us (Arnstein, 1950, 1951) showed that formate, methanol, the β-carbon atom of serine and the α-carbon atom of glycine can be precursors of choline methyl groups. Similar results were reported about the same time by various groups of American workers (Jonsson & Mosher, 1950; Sakami & Welch, 1950; Weissbach, Elwyn & Sprinson, 1950; Du Vigneaud, Verly & Wilson, 1950). Sakami (1950) also demonstrated that the feeding of acetone labelled with ^{14}C in the methyl group gives rise to radioactivity in the methyl group of choline. Du Vigneaud, Verly, Wilson, Rachele, Ressler & Kinney (1951) used methanol labelled both with ^{14}C and deuterium and showed that the hydrogen was diluted to a greater extent than the carbon, indicating that the methyl group of methanol is not directly converted to that of choline. These workers obtained results similar to those reported for formate on feeding [^{14}C]formaldehyde, [^{14}C]formylphenylalanine and [^{14}C]methyl stearate. *In vitro* formation of choline methyl groups from various precursors has also been reported (Sakami & Welch, 1950; Siegel & Lafaye, 1950; Berg, 1951). These experiments and the important observations

on microbe-free rats by Du Vigneaud, Ressler & Rachele (1950; see also Du Vigneaud, Ressler, Rachele, Reyniers & Luckey, 1951) clearly established that the synthesis of methyl groups can occur in the tissues of the rat and need not be ascribed to the activity of the intestinal flora.

As mentioned above, the early experiments of Bennett and her colleagues indicated that certain growth factors, then unidentified, appeared to affect the dietary requirements of the rat for methionine and choline. Evidence has recently been obtained, independently by various workers, that at least one of these factors is cobalamin (Gillis & Norris, 1949; Schaefer, Salmon & Strength, 1949*a*, *b*; Bennett, 1950; Stekol & Weiss, 1950; Jukes & Stokstad, 1951). These studies, which were carried out with rats and chicks, showed that the dietary choline requirement for good growth was reduced and the incidence of kidney damage and mortality in young rats greatly diminished by supplying cobalamin in the diet; however, cobalamin had little beneficial effect if added to diets completely devoid of choline or other compounds containing labile methyl groups. The effect of various levels of choline in the diet on body weight and fatty infiltration of the liver was examined in great detail by Strength, Schaefer & Wilson (1951), and Schaefer & Knowles (1951) who also studied the effects of administration of methylaminoethanol, dimethylaminoethanol, betainė, glycine, serine and methanol on growth.

The aim of the present investigation was to ascertain quantitatively the extent to which synthesis of labile methyl groups, particularly those of choline, takes place in the rat from some of the known precursors mentioned above. In many experiments the radioactivity of the methionine methyl carbon atom was also measured.

The method generally used in tracer experiments consists of administering a labelled compound over a relatively short period. The quantitative interpretation of the results of such experiments requires a detailed knowledge of the nature of the intermediates between the precursor and the isolated product and of the rates of various reactions in which these compounds participate. Such information is not generally available. We have therefore fed over prolonged periods known amounts of labelled compounds as constituents of, in most experiments, synthetic diets. It was hoped that under such conditions, the effects of differences of reaction rates would be largely eliminated. It was also considered desirable to reduce or even eliminate from the diet compounds such as choline and methionine which contain biologically labile methyl groups. The effect of cobalamin in reducing the requirement for choline with purely synthetic diets was therefore investigated; at the same time it was hoped to obtain further information as to the possible relationship between this choline-sparing action of cobalamin and methyl-group synthesis. Some of the results of this investigation have been the subjects of two preliminary communications (Arnstein & Neuberger, 1951, 1952).

EXPERIMENTAL

Animals and diets. Except in Expts. 23 and 24, albino rats of Institute strain were used. The animals were placed on the experimental diets soon after weaning; their ages at the beginning of the experiment were 32 days (Expts. 1–3 and 16–22), 33 days (Expts. 14 and 15), 34 days (Expts. 6, 7 and 11–13; also one rat each in Expts. 16 and 17) and 36 days (Expts. 4, 5 and 8–10) respectively. In Expts. 23 and 24, four albino rats, specially bred from cobalamin-deficient stock, were used. These animals were supplied by Dr W. F. J. Cuthbertson, Glaxo Laboratories, Greenford, Middlesex. Their age at the beginning of the experiment was 46 days and they had been placed on the soya-flour diet (diet 3) described below at 21 days.

To test the efficiency of glycine, serine or formate as methyl-group precursors, one or more litters were distributed as evenly as possible with respect to sex and with respect to the dietary supplement such as cobalamin, choline or methionine. Unless otherwise stated, food consumption was not restricted.

Details of the basal amino-acid diets are given in Table 1. The soya-flour diet (diet 3), kindly supplied by Dr W. F. J. Cuthbertson, had the following composition: soya flour, 72%; lactose, 22%; salt mixture (Hubbell, Mendel & Wakeman, 1937), 4%; arachis oil, 2%; and the following vitamins/kg. diet: vitamin A, 4000 i.u.; vitamin D, 2000 i.u.; vitamin K, 2 mg.; α-tocopherol, 284 mg.; inositol, 220 mg.; nicotinic acid, 100 mg.; calcium D-pantothenate, 100 mg.; *p*-aminobenzoic acid, 75 mg.; thiamine, 30 mg.; riboflavin, 30 mg.; pyridoxine, 8 mg.; pteroylglutamic acid, 1 mg.; biotin, 0·2 mg.

In Expts. 1–3 the amino-acid diet AAD 1 containing 2% [α-^{14}C]glycine (57 μc./mole) was fed throughout the duration of the experiment (39 days). Both choline chloride (5 mg./rat/day) and cobalamin (2·5 μg./rat/day) were given orally. In later experiments (Expts. 4–13) the basal diet AAD 2, containing unlabelled glycine (0·5%), L-serine (0·7%) or sodium formate (0·1%), respectively, was given for an initial period of 12 days; [α-^{14}C]glycine (0·5%; 740 μc/mole), L-[β-^{14}C]serine (0·7%; 220 μc/mole) or sodium [^{14}C]formate (0·1%; 2·8 mc/mole) were then substituted for the respective unlabelled compounds and fed for the remainder of the experiments (20 days, except in the case of one of the two rats used in Expt. 9 which was fed the [β-^{14}C]serine for 16 days only). In Expts. 18–21, diet AAD 2 containing unlabelled glycine (0·5% or 2%) was fed for the first 8 days; 0·5% or 2% [α-^{14}C]glycine (160 μc/mole) were then substituted and fed for 21 days. Expt. 22 was carried out exactly as Expt. 11, except that the specific radioactivity of the sodium [^{14}C]formate fed was 1·1 mc/mole. In Expts. 14 and 15, the diet contained 1% DL-methionine as well as choline chloride (5 mg./rat/day). The animals in these and in Expts. 16 and 17 were pair-fed the respective diets (see Table 3), which contained 0·1% sodium [^{14}C]formate (1·1 mc/mole) throughout the experimental period (28 days).

In the above experiments (Expts. 4–22) cobalamin (2·5 μg./rat/day) was again given orally, but the choline chloride (5 mg./rat/day) was added to the diets daily. In Expts. 23 and 24, the animals, which were pair-fed, received no choline chloride but cobalamin was given both orally (5 μg./rat/day) and in the diet (5 μg./rat/day).

Table 1. *Composition of basal amino-acid diets used in feeding experiments*

(Amounts are given in g./kg. diet. The following vitamins were added to the two diets (mg./kg. diet): α-tocopherol, 40; vitamin K, 1; thiamine, 10; riboflavin, 10; pyridoxine, 10; nicotinic acid, 10; *p*-aminobenzoic acid, 10; calcium pantothenate, 50; inositol, 100; pteroylglutamic acid, 2; biotin, 0·1. 7200 i.u. of vitamin A and 1200 i.u. of vitamin D both/kg. diet were also added.)

Component of diet	Amino-acid diet 1 (AAD 1)	Amino-acid diet 2 (AAD 2)
DL-Valine	12	20
DL-Leucine	18	24
DL-Isoleucine	18	16
L-Arginine monohydrochloride	8	8
L-Histidine monohydrochloride hydrate	7	10
L-Lysine monohydrochloride dihydrate	28	18
DL-Threonine	16	14
DL-Homocystine	12	10
L-Cystine	5	7
DL-Phenylalanine	15	12
L-Tyrosine	10	6
DL-Tryptophan	4	8
[α-^{14}C]Glycine	20	5*
L-Proline	0	4
L-Aspartic acid	0	6
L-Glutamic acid	0	24
$NaHCO_3$	16	13
Maize starch	351	340
Salt mixture (Glaxo Laboratories) no. DL6	40	40
Cane sugar	340	340
Cod-liver oil	16	16
Arachis oil	64	64

* Only some of the animals of this group received [α-^{14}C]glycine, others had instead L-[β-^{14}C]serine or [^{14}C]-formate at levels indicated in the text.

The cobalamin used in all experiments was a highly potent concentrate ('Cytamen', made by Glaxo Laboratories Ltd.) kindly supplied by Dr W. F. J. Cuthbertson.

Food consumption and body weight. The food consumption of each animal was measured daily and the body weight on alternate days.

Radioactivity measurements. All measurements were carried out with a helium-filled bell-shaped Geiger-Müller counter, using 'infinite thickness' samples mounted on 1 sq.cm. Polythene disks (Popják, 1950). The combined errors, due to variation in sample preparation, small differences in disk size and statistical errors in the counting, were about ±5%. All counts were compared with a subsidiary standard and converted to μc. In experiments with synthetic [^{14}C]choline chloroplatinate (Arnstein, 1952) it was found that the back-scattering of the chloroplatinate, compared with $BaCO_3$, was not significant with our instrument and no correction for this effect was made in our calculations.

Isolation and degradation of choline. The animals were killed under anaesthesia and choline was extracted with ethanol separately from the minced pooled viscera (liver, kidney, spleen, reproductive organs, washed gastro-intestinal tract, heart and lungs) and from the minced carcasses respectively. In all experiments the choline (free and bound) was isolated as the reineckate which was converted to the chloroplatinate (Du Vigneaud, Cohn, Chandler, Schenck & Simmonds, 1941). In early experiments it was found that the crude choline chloroplatinate samples sometimes contained some ammonium chloroplatinate, which could be removed by filtration after dissolving the choline chloroplatinate in a little water. The choline chloroplatinate was crystallized from the filtrate by adding ethanol. Repeated purification of the choline chloroplatinate by this method afforded samples of constant radioactivity (Expts. 4–13, 18–21, 23 and 24). In some cases (Expts. 18–21) insufficient material was available for repeated recrystallization and the ammonia content was therefore calculated from the Pt analysis or by estimation using the Kjeldahl method. Later (Expts. 14–17 and 20–22), purification of choline by converting the reineckate into the mercurichloride complex ($C_5H_{14}ONCl.HgCl_2$, m.p. 170°), from which choline chloride was regenerated by H_2S before precipitation as the chloroplatinate, was found to be more satisfactory and to give samples which contained no ammonia as judged by the Kjeldahl method and Pt analysis.

During the course of this work it was found (Arnstein, 1952), that the oxidation of choline chloroplatinate with alkaline permanganate (Du Vigneaud *et al.* 1941) sometimes produces a relatively large amount of ammonia, which is precipitated as the chloroplatinate, together with the trimethylamine. The trimethylamine chloroplatinate obtained by this method was therefore combusted and assayed as $BaCO_3$ (Arnstein, 1952). Some of the values of the radioactivities of choline methyl groups, based on the direct assay of the 'trimethylamine' chloroplatinate, which were reported in one of our preliminary communications (Arnstein & Neuberger, 1951) may therefore be somewhat low. However, our present results show, on the whole, good agreement with our earlier experiments.

Radioactivity estimation of methionine methyl carbon atom and serine β-carbon atom. The ethanol-extracted viscera or carcasses were extracted three times with 6% (w/v) trichloroacetic acid, washed with water, hot ethanol and ether and dried at 80°. For the degradation of methionine, samples of the residue (3–10 g.) were hydrolysed with HI (sp.gr. 1·7; 3·6 ml./g. dry tissue), while N_2 was passed through the solution, essentially as described by Simmonds, Cohn, Chandler & Du Vigneaud (1943). The methyl iodide was absorbed in a 5% (w/v) solution of trimethylamine in methanol, which was cooled in solid CO_2. Crude tetramethylammonium iodide was isolated by evaporating the above solution to dryness. After recrystallization from water by adding ethanol, samples showing no change of radioactivity on further recrystallization were assayed directly for ^{14}C.

In most of the experiments reported in this paper, the radioactivity of the β-carbon atom of serine was measured on the formaldehyde dimedon derivative obtained by periodate oxidation as follows. The ethanol-extracted residues of the viscera were hydrolysed with 6N-HCl for 24 hr. at 100°. The excess HCl was removed and the amino acids were precipitated as Hg complexes (Neuberg & Kerb, 1912; Campbell & Work, 1952). After regeneration with

H_2S, the mixture was oxidized with periodate (Rees, 1946) and the formaldehyde derived from the β-carbon atom of serine was precipitated as the dimedon derivative, which was recrystallized from ethanol to constant radioactivity and unchanged melting point (189°). The validity of this method was checked by comparing the radioactivity of the formaldehyde dimedon obtained as above with that of the dimedon derived from the periodate oxidation (Sakami, 1950) of chromatographically pure serine isolated from the same source (Arnstein & Neuberger, 1953).

RESULTS

The effect on growth of addition of cobalamin and/or suboptimal amounts of choline to basal diets lacking substances with preformed methyl groups

Experiments without restriction of food intake. In the main series of experiments the food intake of the rats was not restricted. Twelve rats altogether (group I) were fed diets which contained cobalamin and homocystine, but were devoid of methionine and choline. The diets contained, in addition to the essential amino acids (except methionine), cystine and one of the labelled substances to be tested, i.e. glycine or serine or formate. Three of the rats which received formate died in the first few days and showed haemorrhagic changes of the kidneys. All other animals survived for the duration of the experiments and appeared to be well. None of the rats which received cobalamin lost weight, but their average body weight increase was only 0·57 g./day (Table 2).

Another group of rats (group II) comprising fourteen animals received the same basal diet as group I, but cobalamin was replaced by choline chloride (5 mg./rat/day). There were no deaths in this group, but *post mortem* examination revealed old lesions in the livers and kidneys of several animals. The average weight gain was only 0·27 g./day and with some of the animals, weight at the end of the experiment was lower than the initial body weight. Statistical analysis (Table 2) suggests that the first diet which contained cobalamin, but no choline, is slightly better than the second diet which contained an insufficient amount of choline but no cobalamin. One animal (Expt. 6), which was not included in calculating the mean values given in Table 2, grew fairly well, the mean daily weight increase being 2·3 g. This was probably due to intestinal synthesis of cobalamin.

The addition of cobalamin to a diet containing the same amount of choline as that given to rats of group II (5 mg./day) resulted in an average weight increase of 1·87 g./day (Table 2). The growth rate found for this group (group III) is still about 30% lower than that observed with rats of the same strain fed a diet containing casein (12%) supplemented with cystine (0·3%) and cobalamin. However, it is clear from the representative growth curves (Fig. 1) and from the statistical analysis (Table 2), that the differences in growth rate and food consumption between group III and groups I and II respectively are highly significant.

The growth rates on the three diets (groups I–III) appeared to be independent of the sex of the animal and not to be related significantly to initial weight. Growth was somewhat better on the second amino-acid diet (AAD 2, Table 1) which was used in most experiments than on amino-acid diet 1 (AAD 1, Table 1). There was no evidence that addition of glycine at the two levels of 2 and 0·5% or of serine affected growth rate or food consumption, but formate at the level of 0·1% had a slight but definite

Table 2. *Effect of administration of cobalamin (2·5 μg./rat/day) or of choline chloride (5 mg./rat/day) or of both together on body weight and food consumption of rats given a basal diet lacking substances containing labile methyl groups*

(For particulars of basal diet see Table 1; experimental periods were between 29 and 39 days; the initial ages of the animals are given in the experimental section.)

Group no.	No. and sex of animals		Presence (+) or absence (−) of		Average initial wt. (g.)		Average daily wt. change (g.)		Average daily food consumption (g.)	
	M.	F.	Cobalamin	Choline	Mean	S.D.	Mean	S.D.	Mean	S.D.
I	5	4	+	−	63	±9·3	+0·57	±0·33	4·88	±1·68
II	9	5	−	+	66	±7·9	+0·27	±0·33	4·59	±1·44
III	11	5	+	+	64·5	±6·2	+1·87	±0·56	7·22	±1·46

	Average daily change in body wt.	Average daily food consumption
Group I *v.* group II	$P<0·05$	$P>0·6$
Group I *v.* group III	$P<0·001$	$P<0·01$
Group II *v.* group III	$P<0·001$	$P<0·001$

P, the probability that difference between dietary groups is due to chance alone, was calculated from Student's tables using as value for the number of degrees of freedom the total number of animals of each pair of groups compared less 2.

growth-retarding effect with all three types of diet.

Experiments involving paired feeding. The average daily food intake of the animals which received both cobalamin and choline (group III) was about 50% greater than that found for either group I or II (Table 2). The increased growth found for rats receiving both supplements as compared with that observed with animals receiving choline alone or cobalamin alone might therefore be largely due to increased food intake. Two series of paired-feeding experiments (Table 3; rats 1–10 and 11–14 respectively) were therefore done: in the first the amino-acid diet (AAD 2) containing also choline and formate was used (Expts. 14–17), whilst in the second series (Expts. 23 and 24) the soya-flour diet was employed. In the first series there was either loss of weight or poor growth and the results were not affected significantly by the presence or absence of cobalamin. In the second series growth was relatively good, but again addition of cobalamin had no effect on weight changes.

It will be noticed that addition of methionine at the level of 1% to a diet already containing homocystine (0·8%) did not improve growth rate (Table 3, rats 1–4). Similar results, suggesting that combined administration of homocystine and methionine may result in depression of growth, have been obtained in other experiments not described here. The reasons for this effect are being investigated.

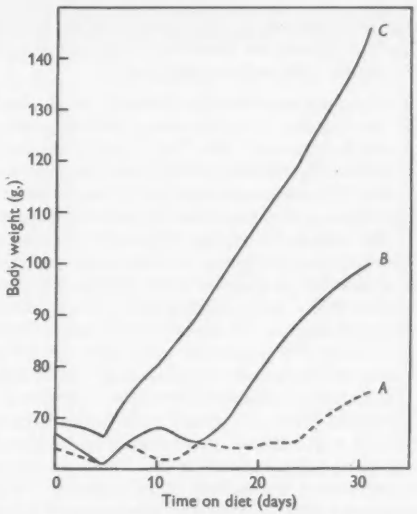

Fig. 1. Representative growth curves of rats on various diets, showing the effect of cobalamin with or without suboptimal amounts of choline. All rats received the basal amino-acid diet AAD 2 (Table 1). Curve *A* is the growth curve of a rat receiving AAD 2 + 5 mg. choline chloride/day without cobalamin; curve *B* gives the data of a litter mate receiving AAD 2 + cobalamin (2·5 μg./day) without choline; curve *C* is the growth curve of another litter mate given AAD 2 with both supplements.

Table 3. *Paired-feeding experiments on the effect of cobalamin on the growth rates of rats receiving various basal diets*

(Rats nos. 1–10 had an amino-acid basal diet (AAD 2, Table 1) containing homocystine; these rats also received sodium [^{14}C]formate (0·1% of the diet) and choline chloride (5 mg./rat/day). Rats nos. 1–4 were also given DL-methionine (1% of the diet) which replaced an equivalent amount of carbohydrate. Rats nos. 11–14 were given the soya-flour diet described in the Experimental section and [α-^{14}C]glycine (1% of the diet). All odd-numbered rats received cobalamin, whilst even-numbered animals received no cobalamin. The bracketed rats were pair-fed in such a way that the food intake of the odd-numbered animal was approx. equal to that of the corresponding even-numbered mate.)

Expt. no.	Rat		Duration of experiment (days)	Body wt. (g.)		Average daily food consumption (g.)
	No.	Sex		Initial	Final	
14	1	M.	17	96	91	5·7
15	2	M.	17	104	83	6·0
14	3	F.	28	92	81	6·2
15	4	F.	28	92	90	6·2
16	5	M.	28	100	88	6·0
17	6	M.	28	97	83	5·9
16	7	M.	28	79	85	6·6
17	8	M.	28	80	97	6·7
16	9	F.	28	77	86	6·9
17	10	F.	28	72	88	6·7
23	11	M.	10	129	152	12·3
24	12	M.	10	115	138	12·3
23	13	M.	10	96	115	11·0
24	14	M.	10	102	120	11·0

Quantitative aspects of the conversion of the α-carbon atom of glycine, the β-carbon atom of serine and of formate to methionine methyl groups

The present experiments (Table 4) show clearly that the extent to which the α-carbon atom of glycine is converted into the methyl groups of methionine is dependent on the level of glycine in the diet. The molar radioactivity of the methionine methyl groups obtained from the internal organs of rats fed a diet containing 2% [α-^{14}C]glycine and cobalamin but no choline or methionine (Expt. 1), was about 13% of that of the fed glycine. The latter had, however, been diluted by glycine formed through endogenous synthesis and it can be calculated from the observed radioactivity of the glycine of the internal organs that the dilution factor in this experiment was 2·9 (Arnstein & Neuberger, 1953). It would thus appear that at a dietary level of 2% of glycine about 35–40% of the methionine methyl groups of the internal organs are derived from the α-carbon atom of glycine. When the glycine level in the diet was only 0·5%, the molar radioactivity of the methionine methyl groups of the internal organs was less than 2% of that of the fed glycine (Expt. 4). In this experiment the dilution of the fed glycine by glycine formed through endogenous synthesis was about 8·0 (Arnstein & Neuberger, 1953) and thus, with this lower level of glycine, only about 14% of methyl groups are derived from the α-carbon atom of glycine.

When L-[β-^{14}C]serine was fed at a level of 0·7%, which is equivalent on a molar basis to 0·5% glycine, the molar radioactivity in the methionine methyl groups (Expt. 8) was approximately 6 times that observed in the comparable glycine experiment (Expt. 4) and was about 70% of that of the β-carbon atom of serine isolated from the internal organs of the same animals.

Sodium [^{14}C]formate was fed at a level of 0·1%, equivalent to 0·16% of serine. The results are therefore not strictly comparable, but it appears (Expt. 11) that its conversion into methionine methyl groups is at least as efficient as that of the β-carbon atom of serine.

In the experiments so far considered, no compounds containing labile methyl groups were present in the diet. When choline chloride was fed at amounts of 5 mg./rat/day, in addition to cobalamin, the radioactivity of the methionine methyl groups was reduced by 25–30% (Expts. 5, 9 and 12) as compared with similar experiments in which cobalamin but no choline was supplied (Expts. 4, 8 and 11). On

Table 4. *The incorporation of radioactivity from* [α-^{14}C]*glycine,* L-[β-^{14}C]*serine and sodium* [^{14}C]*formate into the β-carbon atom of serine and the methyl carbon atom of methionine*

(For details of diets see Table 1 and the Experimental section. The average daily food consumption and growth of the animals are given in Table 2. The β-carbon atom of serine and the methionine methyl carbon atom were isolated from the hydrolysates of the mixed viscera proteins, as described in the Experimental section. In order to facilitate comparison, the specific radioactivities of the β-carbon atom of serine and the methionine methyl carbon atom are expressed as μc/g. atom and have been calculated for a specific radioactivity of labelled precursor in the diet = 1 mc/mole.)

Expt. no.	Basal diet	Labelled precursor	Supplement	Specific radioactivity (μc/g. atom): Serine β-carbon atom	Specific radioactivity (μc/g. atom): Methionine methyl carbon atom
1	AAD 1	2% [α-^{14}C]Glycine	Cobalamin	234	128
2			Choline + cobalamin	226	—
3			Choline	239	—
4	AAD 2	0·5% [α-^{14}C]Glycine	Cobalamin	33·6	17·8
5			Choline + cobalamin	30·1	11·0
6*			Choline	32·2	13·7
7			Choline	32·0	—
8	AAD 2	0·7% L-[β-^{14}C]Serine	Cobalamin	144	107
9			Choline + cobalamin	153	81·5
10			Choline	129	47·4
11	AAD 2	0·1% Sodium [^{14}C]formate	Cobalamin	9·4	40·6
12			Choline + cobalamin	7·0	33·8
13			Choline	6·4	20·0
14†			1% Methionine, choline + cobalamin	—	10·3
15†			1% Methionine, choline	—	8·3
16†			Choline + cobalamin	—	32·0
17†			Choline	—	28·6

— Signifies values not determined.

* The animal in this experiment grew abnormally well, possibly due to intestinal synthesis of cobalamin.

† The animals in these experiments were pair-fed.

diets containing choline chloride (5 mg./rat/day) but no cobalamin, the radioactivity of the methionine methyl groups was further reduced (Expts. 10 and 13). In one experiment (Expt. 6) there was no significant difference, but this animal grew exceptionally well (see p. 262) and it is presumed that extensive intestinal synthesis of cobalamin took place. This effect of cobalamin of increasing the radioactivity of methionine methyl groups was markedly reduced, but still significant, in experiments in which groups of rats were pair-fed (Expts. 14–17, Table 4; Expts. 23 and 24, Table 7) even when an adequate amount of methionine was given in the diet (Expts. 14 and 15, Table 4; Expts. 23 and 24, Table 7).

Table 4 also gives data for the radioactivity of the β-carbon atom of serine of the visceral proteins. In all experiments in which [α-^{14}C]glycine or [β-^{14}C]-serine were given, the serine β-carbon has a radioactivity which is 1·3 to 3·0 times that of methionine methyl carbon atom. By contrast, labelled formate gives rise to a radioactivity in the β-carbon atom of serine which is one-third to one-fifth that of the methionine methyl carbon atom.

In the glycine and serine experiments (nos. 1–10) the specific radioactivity of the β-carbon atom of serine was not affected significantly by administration of choline or cobalamin, but with formate as precursor addition of choline may cause a reduction in radioactivity (Expts. 11–13). It is also of interest that at the two levels of glycine the ratios of radioactivities of the β-carbon atom of serine to that of the methionine methyl groups are similar, but slightly lower than those found in comparable experiments in which [β-^{14}C]serine was the labelled precursor.

Comparison of the incorporation of radioactivity from [α-^{14}C]*glycine,* [β-^{14}C]*serine and sodium* [^{14}C]-*formate into choline methyl groups*

Tables 5 and 6 show the radioactivity of choline and the relative incorporation of ^{14}C into choline methyl groups from [α-^{14}C]glycine, [β-^{14}C]serine and [^{14}C]formate under various conditions. The [α-^{14}C]-glycine was fed at two different levels and, since results have been calculated on the basis of a uniform molar radioactivity of precursor (1 mc/mole), the specific radioactivity of the choline isolated from animals which had been fed glycine at a dietary level of 2% (Expts. 18 and 19) was, as expected, several times higher than in corresponding experiments in which 0·5% glycine was fed. The same applies to the specific radioactivity of the ethanolamine moiety of choline, which can be calculated by difference. The radioactivity of the trimethylamine moiety was affected to a greater extent than can be accounted for solely by the greater intake of radioactive precursor on the 2% glycine diet. This result is similar to the findings on the conversion of [α-^{14}C]glycine into methionine methyl groups described above.

At the 2% glycine level the administration of cobalamin appears to increase specifically the incorporation of radioactivity into the choline methyl groups (Expts. 18 and 19), which confirms our earlier findings (Arnstein & Neuberger, 1951). In a similar experiment, in which animals were pair-fed a diet containing 1% [α-^{14}C]glycine, in addition to an unknown amount of unlabelled glycine derived from the soya-flour protein (Table 7) for a shorter period (10 days), the addition of cobalamin increased the relative radioactivity in the choline methyl groups from 12 to 21%. However, this effect of cobalamin is not marked on the 0·5% glycine diet (Table 5; Expts. 5, 20 and 21).

The β-carbon atom of serine, fed at a level equivalent to 0·5% glycine, appears to be more effective as a precursor of the ethanolamine moiety of choline than the α-carbon atom of glycine. The incorporation of ^{14}C into the methyl groups is even more greatly increased, as indicated by the observation that in these experiments (nos. 9 and 10) more than 50% of the radioactivity of the choline is present in the trimethylamine moiety.

Formate, on the other hand, appears to be incorporated mainly into the methyl groups of choline, which contain between 60 and 85% of the total radioactivity.

Tables 5 and 6 also show that the radioactivity of the choline is reduced by about 50% when 5 mg. of choline chloride/day are supplied. A comparison of experiments in which animals received choline and cobalamin with those in which choline only was supplied shows a marked increase of choline synthesis from the labelled precursors when this vitamin is given. In paired feeding experiments using [^{14}C]formate (nos. 14–17) this effect of cobalamin, though less marked in the case of diets containing an adequate amount of methionine (Expts. 14 and 15), was still present.

In all experiments, with the exception of the high-glycine diets discussed above, the relative incorporation of ^{14}C from the labelled precursor into choline methyl groups compared with the ethanolamine moiety was hardly affected by the administration of cobalamin, indicating that the latter stimulated conversion of the labelled carbon atoms into the two moieties to an approximately equal extent.

It is of interest that although the labelled precursors were fed over periods of as long as 5 weeks the radioactivity of the carcass choline was always considerably less than that of the visceral choline (Tables 5 and 6). Differences of the same order were observed for the methionine methyl groups derived from carcass and visceral proteins (Table 7), but in

Table 5. *Conversion of the α-carbon atom of glycine and the β-carbon atom of* L-*serine into choline*

(The composition of the basal diet (AAD 2) which was used in these experiments is given in Table 1. Details of the dietary supplements, the food consumption and growth of the animals are given in Table 2 and in the Experimental section. The choline was isolated either separately from the pooled viscera and from the carcass or from the total body. The isolation and degradation methods are described in the Experimental section. The specific radioactivities of the isolated compounds have been calculated for a specific radioactivity of precursor = 1 mc/mole in order to facilitate comparison of different experiments.)

Expt. no.	Labelled precursor	Supplement to basal diet	Specific radioactivity (μc/mole) of choline* isolated from: Viscera	Carcass	Total body	Specific radioactivity (μc/mole) of trimethylamine obtained by degradation of choline from: Viscera	Carcass	Total body	Percentage of radioactivity of choline in methyl groups: Viscera	Carcass	Total body
18	2% [α-^{14}C]Glycine	Choline + cobalamin	512[a]	326[b]	—	208	135	—	41	41	—
19		Choline	182[a]	88·0[b]	—	37·4	26·2	—	21	30	—
4	0·5% [α-^{14}C]Glycine	Cobalamin	—	—	103[b]	—	—	24·0	—	—	23
5		Choline + cobalamin	—	—	47·6[b]	—	—	8·5	—	—	18
20		Choline + cobalamin	119[d]	86·0[a]	—	31·8	20·6	—	27	24	—
6†		Choline	—	—	44·8[b]	—	—	10·4	—	—	23
7		Choline	—	—	23·1[b]	—	—	—	—	—	—
21		Choline	59·3[d]	25·6[c]	—	9·4	6·2	—	16	24	—
8	0·7% L-[β-^{14}C]Serine	Cobalamin	—	—	217[b]	—	—	—	—	—	—
9		Choline + cobalamin	—	—	110[b]	—	—	61·8	—	—	56
10		Choline	—	—	69·5[b]	—	—	36·0	—	—	52

— Signifies value not determined.

* The radioactivity of the choline was calculated from the assay of the chloroplatinate (see Experimental section) as follows:
(*a*) After correction for the ammonium chloroplatinate content, as determined by Pt analysis.
(*b*) After recrystallization of the choline chloroplatinate to constant radioactivity.
(*c*) After correction for the ammonium chloroplatinate content, as determined by Pt analysis and Kjeldahl estimation.
(*d*) After purification of the choline through the $HgCl_2$ complex.

† The animal in this experiment grew abnormally well, possibly due to intestinal synthesis of cobalamin.

Table 6. *Conversion of formate to choline*

(In all the experiments, diet AAD 2 described in Table 1 containing sodium [^{14}C]formate (0·1%) was fed (see Experimental section). The choline was isolated either separately from the pooled viscera and carcass or from the total body. The isolation and degradation methods used are given in the Experimental section. The specific radioactivities of the choline and trimethylamine have been calculated for a specific radioactivity of precursor = 1 mc/mole in order to facilitate comparison of different experiments.)

Expt. no.	Supplements	Specific radioactivity (μc/mole) of choline* isolated from: Viscera	Carcass	Total body	Specific radioactivity (μc/mole) of trimethylamine obtained by degradation of choline from: Viscera	Carcass	Total body	Percentage of radioactivity of choline in methyl groups: Viscera	Carcass	Total body
11	Cobalamin	—	—	47·5[a]	—	—	—	—	—	—
22	Cobalamin	89·0[b]	64·8[b]	—	67·2	46·2	—	75	71	—
12	Choline + cobalamin	—	—	23·1[a]	—	—	18·8	—	—	81
13	Choline	—	—	9·3[a]	—	—	7·85	—	—	84
14†	1% Methionine + choline	19·3[b]	15·1[b]	—	—	10·5	—	—	58·5	—
	+ cobalamin	18·0[b]‡	11·2[b]‡	—	—	6·55‡	—	—	69·5‡	—
15†	1% Methionine + choline	16·2[b]	11·6[b]	—	—	7·65	—	—	66	—
		11·6[b]‡	—	—	—	—	—	—	—	—
16†	Choline + cobalamin	52·5[b]	37·0[b]	—	39·9	22·8	—	76	62	—
17†	Choline	31·4[b]	18·8[b]	—	25·7	16·0	—	82	85	—

— Signifies value not determined.

* The radioactivity of the choline was calculated from the assay of the chloroplatinate (see Experimental section) as follows: (*a*) After recrystallization of the choline chloroplatinate to constant radioactivity. (*b*) After purification of the choline through the $HgCl_2$ complex.

† The animals in these experiments were pair-fed (see Table 3).

‡ Sample isolated from animal which was killed one week earlier than the other animals in this experiment.

Table 7. *Conversion of the α-carbon atom of glycine into choline and into the methyl group of methionine under paired-feeding conditions*

(In these experiments four rats, specially bred from cobalamin-deficient stock, were pair-fed (see Table 3) for 10 days a soya-flour diet (diet 3, Experimental section), which contained 1% [α-^{14}C]glycine. The methyl carbon atom of the methionine, derived from the proteins of either the pooled viscera or the carcass, and the choline from the total body were isolated as described in the Experimental section and recrystallized to constant radioactivity. The specific radioactivities of the compounds isolated have been calculated for a specific radioactivity of precursor = 1 mc/mole in order to facilitate comparison with other experiments.)

Expt. no.	Supplement given	Specific radioactivity (μc/mole or μc/g. atom): Methionine methyl carbon atom from: Viscera	Carcass	Choline (from total body)	Trimethylamine moiety of choline	Percentage of radioactivity of choline in methyl groups
23	Cobalamin (5 μg./day added to diet + 5 μg./day orally)	7·4	3·4	34·4	7·1	21
24	None	5·9	3·2	35·4	4·4	12

this case the experimental period was relatively short (10 days) and the results are therefore not strictly comparable.

DISCUSSION

The effect of cobalamin on the growth of rats fed diets deficient in substances containing labile methyl groups

The growth rates obtained in experiments in which food intake was not restricted (Table 2) are in general agreement with those of Schaefer & Knowles (1951) who used diets containing peanut meal and oxidized casein, and those of Stekol & Weiss (1950) who used amino-acid diets similar to those employed in the present work. A diet containing cobalamin, homocystine and cystine, but no methionine or choline, was found consistently to support very slow growth. On the other hand, a diet of similar composition, but in which cobalamin was replaced by a suboptimal amount of choline chloride (5 mg./rat/day) was less regular in its effect; some animals lost weight, whilst others grew at a very slow rate. Combined administration of the same suboptimal amount of choline and of cobalamin produced moderately good growth. It would appear that the supply of 2·5 μg. of cobalamin in our experiments is equivalent to the administration of somewhat more than 5 mg. of choline chloride. We have also found, confirming the observations of Schaefer & Knowles (1951), that the addition to the diet of serine, glycine

or formate, substances which are known to be precursors of labile methyl groups, has no stimulating effect on the growth with any of the diets used.

The growth-promoting effect of cobalamin was absent in experiments in which paired feeding was used (Table 3). Similar observations showing that the marked effect of cobalamin on the growth of rats can be largely attributed to increased food intake have recently been reported by Black & Bratzler (1952) who used somewhat different diets.

The relative importance of glycine, serine and formate as precursors of methyl groups

As already stated above, the labelled substances were fed over relatively long periods in the hope that a steady state would be reached at the end of the experiment which would enable us to assess quantitatively the contributions made by the various precursors to the synthesis of methyl groups. The concept of the steady state implies that the turnover and 'mixing' times of all components of the system under consideration are infinitely small compared with the period of the experiment. Under such conditions the isotope content of a reaction product compared with that of one of its precursors should yield a quantitative measure of the proportion of the product derived from the given precursor.

The present results indicate that this steady state has not been reached, even in experiments in which the labelled compound was administered for 28 days. Thus the isotope content of the choline derived from the viscera always exceeded that of the choline obtained from the carcass, the differences varying between 25 and 100% (Tables 5 and 6). Similar differences were also observed between the glycine isolated from the viscera and carcass proteins respectively (Arnstein & Neuberger, 1953). It thus appears that equilibration between the internal organs and the rest of the body has not been achieved. However, in studies on the metabolism of proteins and other constituents of the internal organs, such as liver and kidney, turnover times have been found which were appreciably shorter than the experimental periods used in most of the present work. It may be accepted therefore that a comparison of isotope data based on results obtained with compounds isolated from the viscera should yield at least semiquantitative conclusions about the metabolic relationships under investigation.

In all experiments the isotope content of the methionine methyl groups was appreciably higher than that of the choline methyl groups (Table 8). The most reasonable explanation of this finding is that the various precursors give rise, in the first place, to methyl groups in methionine and that these are then incorporated into choline by transmethylation. Such an interpretation agrees with the findings of Berg (1951) who used *in vitro* systems. The alternative explanation that the methyl groups are incorporated independently into both choline and methionine and that the former has a much

Table 8. *Comparison of the specific radioactivity of the methyl groups of methionine and choline*

(Details of the isolation and degradation of choline and methionine and of the radioactivity determinations are given in the Experimental section. The specific radioactivity of the methyl groups is expressed as μc/g. atom, calculated for a specific radioactivity of precursor = 1 mc/mole.)

				Specific radioactivity (μc/g. atom)				
				Choline methyl carbon atom			Methionine methyl carbon atom	
Expt. no.	Basal diet	Labelled precursor	Supplement	Viscera	Carcass	Total body	Viscera	Carcass
1	AAD 1	2% [α-^{14}C]Glycine	Cobalamin	—	—	—	128	—
18	AAD 2		Choline + cobalamin	69	45	—	—	—
19	AAD 2		Choline	12·5	8·7	—	—	—
23*	Diet 3	1% [α-^{14}C]Glycine	Cobalamin	—	—	2·4	7·4	3·4
24*	Diet 3		None	—	—	1·5	5·9	3·2
4	AAD 2	0·5% [α-^{14}C]Glycine	Cobalamin	—	—	8·0	17·8	—
5	AAD 2		Choline + cobalamin	—	—	2·8	11·0	—
6†	AAD 2		Choline	—	—	3·5	13·7	—
9	AAD 2	0·7% L-[β-^{14}C]Serine	Choline + cobalamin	—	—	20·6	81·5	—
10	AAD 2		Choline	—	—	12·0	47·4	—
12	AAD 2	0·1% Sodium [^{14}C]formate	Choline + cobalamin	—	—	6·3	33·8	—
13	AAD 2		Choline	—	—	2·6	20·0	—
14*	AAD 2		1% Methionine + choline + cobalamin	—	2·2	—	10·3	—
15*	AAD 2		1% Methionine + choline	—	3·5	—	8·3	—
16*	AAD 2		Choline + cobalamin	13·3	7·6	—	32·0	—
17*	AAD 2		Choline	8·6	5·3	—	28·6	—

* The animals in these experiments were pair-fed.
† The animal in this experiment grew abnormally well (see footnote, Table 4).

slower turnover rate, at least under conditions of a dietary methyl-group deficiency, is not excluded. Table 4 also shows clearly that formate differs strikingly from [β-^{14}C]serine and [α-^{14}C]glycine, in giving rise to a relatively low activity in the β-carbon atom of serine and a relatively high activity in the methionine methyl carbon atom. It would follow that the conversion of formate into methyl groups cannot proceed by a pathway involving serine as an intermediate. The earlier suggestion made by one of us (Arnstein, 1951) and based on short-term experiments, that serine is concerned in this transformation, is therefore incorrect. The radioactivity of the methionine methyl groups after feeding labelled formate was high (Table 4), taking into account the relatively small amount of formate supplied and the fact that the results are calculated on the basis of a constant radioactivity of substance administered. The significance of this observation is difficult to assess, as it is still uncertain whether formate itself is an important physiological intermediate. Moreover, the extent to which the dietary formate was diluted by endogenous 'formate' is not accurately known. Weinhouse & Friedmann (1952) have estimated that endogenous formation of 'formate' by the rat may be of the order of 0·5–1·5 m-moles/day. A calculation based on these figures would suggest that the importance of formate as precursor of methyl groups is of the same order as that of serine.

In the case of serine, however, the degree to which the dietary amino acid was diluted by endogenous synthesis can be assessed by comparison of the radioactivities of the fed serine and of the serine isolated from the viscera (Arnstein & Neuberger, 1953). It can be deduced that, in the absence of dietary methyl groups and in the presence of cobalamin, about 70–75% of the methionine methyl groups of the proteins of the viscera is derived from the β-carbon atom of serine. This calculation is based on the assumption that the amino acid used for methyl group synthesis is derived from the same 'pool' as that incorporated into proteins of the viscera and that a steady state has been reached in the viscera.

The efficiency of the α-carbon atom of glycine as methyl-group precursor depends on its level in the diet (Tables 4 and 5); it increases with the level as does conversion of glycine to the β-carbon atom of serine (Arnstein & Neuberger, 1953). Moreover, the ratio of specific activities of the methionine methyl carbon atom to that of the β-carbon atom of serine was similar to that found on feeding [β-^{14}C]-serine. It would thus seem that the conversion of the α-carbon atom of glycine into methionine methyl groups proceeds through the β-carbon atom of serine.

The results of the present investigation, together with the observations of other workers, are compatible with the following general scheme. The immediate precursor (X) of methyl groups is a compound or group of compounds related to, or, less likely, identical with, formaldehyde or formate. X may be derived from a variety of sources such as acetone, methanol, histidine (Reid & Landefeld, 1951; Soucy & Bouthillier, 1951; Toporek, Miller & Bale, 1952), tryptophan (Knox & Mehler, 1950), serine or glycine; but some of these precursors may not be quantitatively important under physiological conditions. The present work suggests that the β-carbon atom of serine is quantitatively the most significant of these precursors; this is presumably due to the fact that serine is, unlike histidine or tryptophan, a non essential amino acid and is synthesized in relatively large quantities by the rat. Since some of these precursors, especially serine, are readily available to the animal, the rate-determining step in methyl-group synthesis must be the conversion of the precursors into X or that of X into methyl groups.

The present results would be quantitatively compatible with the view that formate, or a close derivative, is the intermediate (X) in the conversion of the β-carbon atom of serine to labile methyl groups; however, the findings of Elwyn, Weissbach & Sprinson (1951) suggest that formate is not an intermediate in this reaction and we have therefore postulated that different one-carbon intermediates may be concerned in methyl-group synthesis from formate and serine.

The effect of cobalamin on methyl-group synthesis

The choline- or methionine-sparing effect of cobalamin found with rats kept on diets deficient in substances containing labile methyl groups is suggestive evidence, but does not constitute a rigorous proof that cobalamin is concerned with methyl-group synthesis. The present experiments lend further weight to this hypothesis. Rats not supplied with cobalamin synthesized labile methyl group to a significant extent (Tables 4–7), but this may be due to the persistence of cobalamin stores laid down before the dietary restriction was imposed or to microbial synthesis of the vitamin in the intestine. Addition of cobalamin to the deficient diet greatly increased the isotope contents of the methyl groups of both methionine and choline (Tables 4–8) independently of the nature of the labelled precursor, provided food intake was not restricted. In the paired-feeding experiments (Tables 4, 6 and 7) this effect of cobalamin was reduced, but still present, indicating that the effect of cobalamin of increasing the incorporation of labelled carbon into methyl groups cannot be explained entirely as being due to increased food consumption leading to enhanced rate of growth.

The observation that this effect of cobalamin was found with all three precursors suggests that the reaction catalysed by cobalamin is either similar for or common to the various precursors and represents the rate-determining step discussed above. The finding that the ratio between the specific radioactivity of the choline and that of the methionine methyl carbon atom is not markedly affected by cobalamin (Table 8) excludes the possibility that this vitamin is concerned with transmethylation.

The distribution of radioactivity within the choline molecule

The relative amount of the total radioactivity of choline present in the methyl groups appears to be relatively constant and characteristic of the labelled precursor fed. Thus with formate 60–85 % of the total radioactivity was found in the trimethylamine moiety and this ratio was not significantly affected by the presence of cobalamin in the diet (Table 6). With [β-^{14}C]serine the activities in the two fragments of choline were about equal and again there was no marked effect of cobalamin (Table 5). These ratios are not easy to explain in the absence of any detailed knowledge of the turnover rate of choline, the speed of transmethylation from methionine and possible re-utilization of methyl groups. The lack of effect of cobalamin on the ratios, at least with serine and formate as precursors, may seem surprising. It might have been expected that, with the absence of cobalamin and consequent diminution of methyl-group synthesis, the undiminished transformation of serine to ethanolamine should lead to a relative decrease of the radioactivity in the trimethylamine moiety of choline. However, it is possible that, in the absence of cobalamin, very little choline is synthesized, by transmethylation or otherwise, and the utilization of the labelled ethanolamine is therefore reduced. But the possibility that cobalamin is also concerned in the conversion of serine to ethanolamine cannot be completely excluded.

When glycine was fed at a level of 0·5 %, the proportion of the radioactivity present in the trimethylamine moiety was about 20 % and again there was no effect of cobalamin (Table 5). But with a level of 1 % (Table 7) or 2 % (Table 5) the relative radioactivity in the methyl groups was definitely increased by cobalamin. This finding led us to suggest (Arnstein & Neuberger, 1952) that this vitamin stimulates the transformation of glycine into serine and the same idea was put forward by Stekol, Weiss & Weiss (1952). However, the direct measurement of the radioactivity of the β-carbon atom of serine (Table 4; see also Arnstein & Neuberger, 1953) gives no support to this hypothesis. The synthesis and metabolism of choline is particularly complicated owing to the intricate inter-relationship between serine, ethanolamine, methyl-group synthesis and transmethylation. With methionine methyl groups the position is simpler and the conclusion reached in the present work that cobalamin affects methyl-group synthesis is mainly based on the methionine results, particularly those where possible effects of different growth rates were eliminated by paired feeding.

SUMMARY

1. The conversion of the β-carbon atom of serine, the α-carbon atom of glycine and the carbon atom of formate into the methyl groups of choline and methionine has been studied quantitatively by feeding completely defined diets containing these precursors, suitably labelled with ^{14}C, to young rats over prolonged periods.

2. In all experiments the radioactivity of the methionine methyl groups greatly exceeded that of the choline methyl groups, suggesting that most of the newly synthesized methyl groups are first incorporated into methionine and are then transferred to choline by transmethylation.

3. With diets devoid of preformed methyl groups, but containing [β-^{14}C]serine and cobalamin, the methyl group of the visceral methionine had 70 % of the radioactivity of the β-carbon of serine isolated from the same source. This indicates that the hydroxymethyl group of serine is quantitatively the most important precursor of synthesized methyl groups.

4. In similar experiments with [α-^{14}C]glycine, the α-carbon atom of glycine was found to be relatively inefficient as a precursor of methyl groups, particularly when the level of glycine in the diet was not unduly high.

5. Formate was found to be an efficient precursor of methyl groups, but its conversion to the hydroxymethyl group of serine was relatively poor. The results are compatible with the assumption that formate is extensively converted to a substance which is an intermediate in the synthesis of methyl groups from serine.

6. Cobalamin increased the conversion of all three precursors into the methyl groups of methionine and into both the methyl groups and the ethanolamine moiety of choline. The stimulating effect of cobalamin on methyl-group synthesis was reduced, but still present, in paired-feeding experiments. With diets containing 2 % [α-^{14}C]glycine, cobalamin increased the relative incorporation of isotope into the methyl groups of choline, compared with that into the ethanolamine moiety.

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REFERENCES

Arnstein, H. R. V. (1950). *Biochem. J.* **47**, xviii.
Arnstein, H. R. V. (1951). *Biochem. J.* **48**, 27.
Arnstein, H. R. V. (1952). *J. chem. Soc.* p. 4527.
Arnstein, H. R. V. & Neuberger, A. (1951). *Biochem. J.* **48**, ii.
Arnstein, H. R. V. & Neuberger, A. (1952). *Biochem. J.* **50**, xxxviii.
Arnstein, H. R. V. & Neuberger, A. (1953). *Biochem. J.* **55**, 271.
Bennett, M. A. (1950). *J. biol. Chem.* **187**, 751.
Bennett, M. A., Medes, G. & Toennies, G. (1944). *Growth*, **8**, 59.
Bennett, M. A. & Toennies, G. (1946). *J. biol. Chem.* **163**, 235.
Berg, P. (1951). *J. biol. Chem.* **190**, 31.
Black, A. & Bratzler, J. W. (1952). *J. Nutr.* **47**, 159.
Campbell, P. N. & Work, T. S. (1952). *Biochem. J.* **50**, 449.
Du Vigneaud, V., Chandler, J. P., Moyer, A. W. & Keppel, D. M. (1939). *J. biol. Chem.* **131**, 57.
Du Vigneaud, V., Cohn, M., Chandler, J. P., Schenck, J. R. & Simmonds, S. (1941). *J. biol. Chem.* **140**, 625.
Du Vigneaud, V., Ressler, C. & Rachele, J. R. (1950). *Science*, **112**, 267.
Du Vigneaud, V., Ressler, C. & Rachele, J. R., Reyniers, J. A. & Luckey, T. D. (1951). *J. Nutr.* **45**, 361.
Du Vigneaud, V., Simmonds, S., Chandler, J. P. & Cohn, M. (1945). *J. biol. Chem.* **159**, 755.
Du Vigneaud, V., Verly, W. G. & Wilson, J. E. (1950). *J. Amer. chem. Soc.* **72**, 2819.
Du Vigneaud, V., Verly, W. G. L., Wilson, J. E., Rachele, J. R., Ressler, C. & Kinney, J. M. (1951). *J. Amer. chem. Soc.* **73**, 2782.
Elwyn, D., Weissbach, A. & Sprinson, D. B. (1951). *J. Amer. chem. Soc.* **73**, 5509.
Gillis, M. B. & Norris, L. C. (1949). *J. biol. Chem.* **179**, 487.
Hubbell, R. B., Mendel, L. B. & Wakeman, A. J. (1937). *J. Nutr.* **14**, 273.
Jonsson, S. & Mosher, W. A. (1950). *J. Amer. chem. Soc.* **72**, 3316.
Jukes, T. H. & Stokstad, E. L. R. (1951). *J. Nutr.* **43**, 459.
Knox, W. E. & Mehler, A. H. (1950). *J. biol. Chem.* **187**, 419.
Neuberg, C. & Kerb, J. (1912). *Biochem. Z.* **40**, 498.
Popják, G. (1950). *Biochem. J.* **46**, 560.
Rees, M. W. (1946). *Biochem. J.* **40**, 632.
Reid, J. C. & Landefeld, M. O. (1951). *Arch. Biochem. Biophys.* **34**, 219.
Sakami, W. (1950). *J. biol. Chem.* **187**, 369.
Sakami, W. & Welch, A. D. (1950). *J. biol. Chem.* **187**, 379.
Schaefer, A. E. & Knowles, J. L. (1951). *Proc. Soc. exp. Biol., N.Y.*, **77**, 655.
Schaefer, A. E., Salmon, W. D. & Strength, D. R. (1949*a*). *Proc. Soc. exp. Biol., N.Y.*, **71**, 193.
Schaefer, A. E., Salmon, W. D. & Strength, D. R. (1949*b*). *Proc. Soc. exp. Biol., N.Y.*, **71**, 202.
Siegel, I. & Lafaye, J. (1950). *Proc. Soc. exp. Biol., N.Y.*, **74**, 620.
Simmonds, S., Cohn, M., Chandler, J. P. & Du Vigneaud, V. (1943). *J. biol. Chem.* **149**, 519.
Soucy, R. & Bouthillier, L. P. (1951). *Rev. canad. Biol.* **10**, 290.
Stekol, J. A. & Weiss, K. (1950). *J. biol. Chem.* **186**, 343.
Stekol, J. A., Weiss, S. & Weiss, K. W. (1952). *Arch. Biochem. Biophys.* **36**, 5.
Strength, D. R., Schaefer, A. E. & Wilson, W. D. (1951). *J. Nutr.* **45**, 329.
Toporek, M., Miller, L. L. & Bale, W. F. (1952). *J. biol. Chem.* **198**, 839.
Weinhouse, S. & Friedmann, B. (1952). *J. biol. Chem.* **197**, 733.
Weissbach, A., Elwyn, D. & Sprinson, D. B. (1950). *J. Amer. chem. Soc.* **72**, 3316.

The Synthesis of Glycine and Serine by the Rat

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It has been known for almost 50 years that glycine can be synthesized by mammals. Thus Wiechowski (1906) and Magnus-Levy (1907) showed that animals fed benzoate over long periods excreted more hippuric acid than could be accounted for either by the glycine present in their body proteins or that ingested. Later, McCoy & Rose (1937) and Rose, Burr & Sallach (1952) using diets containing pure amino acids demonstrated clearly that neither glycine nor serine has to be supplied to the young rat in order to obtain a good rate of growth. It has also been shown that other mammalian species, including man, can synthesize amounts of glycine sufficient to maintain body weight and nitrogen equilibrium and to support a normal growth rate. The hen also can synthesize glycine, but not at a rate permitting optimum growth, as shown by the fact that growth can be greatly improved by addition of glycine to a diet deficient in this amino acid (Almquist, Stokstad, Mecchi & Manning, 1940; Almquist & Grau, 1944). The turkey, too, requires glycine for optimum growth (Jukes, Stokstad & Belt, 1947), although the amounts needed appear to be lower than in the hen (Kratzer & Williams, 1948). It thus appears likely that glycine is a 'semi-essential' amino acid for all birds.

Even in mammals, there is good evidence that the rate of synthesis of glycine cannot greatly surpass that required by the demands of growth. Thus it is uncertain whether the growth inhibition and general toxicity of benzoate are entirely due to the removal from the body of glycine in the form of hippurate; but the addition of glycine to the diet abolishes the toxic effects of benzoate and restores the growth rate to a normal value (Griffith, 1929; White, 1941), indicating clearly that the rate of synthesis of this amino acid in the rat is not large enough to supply sufficient glycine both for growth and for conjugation of quantities of benzoate of 1–2 m-moles per day. Similar conclusions can be drawn from the earlier observations of Griffith & Lewis (1923) and Csonka (1924) on the effect of the glycine content of the diet on the extent of hippuric acid excretion after a given dose of benzoate. Experiments of this type do not, however, permit any quantitative conclusions concerning the rate of synthesis of glycine under normal conditions, and it was expected that the use of isotope methods would afford a more satisfactory approach to this problem.

In experiments primarily designed for another purpose (Arnstein & Neuberger, 1953) known amounts of glycine and serine were fed to young rats over relatively long periods. It was thought that a comparison of the radioactivity of the fed amino acid with that of the amino acid isolated from the tissues of the animal at the end of the experiment should yield information of the amounts of these two amino acids synthesized by the rat. The effect of the amount of glycine in the diet on the rate of synthesis was also examined and this also afforded an opportunity to study some quantitative aspects of the conversion of glycine to serine, particularly that of the α-carbon of the former to the β-carbon of the latter.

EXPERIMENTAL

Animals and diet. Albino rats of Institute strain were placed on the experimental diets soon after weaning, with the exception of Expt. 2, in which adult animals were used.

The amino-acid diets AAD 1 and AAD 2 have been described previously (Arnstein & Neuberger, 1953). The casein diet used in Expts. 1–3 had the following composition (g./kg. diet): [α-^{14}C]glycine, 20; casein (light, white, unextracted), 125; salt mixture (Glaxo Laboratories, no. DL 6), 40; cod-liver oil, 16; arachis oil, 64; cane sugar, 340; maize starch, 395. Where indicated, cobalamin (2·5 μg./rat/day) was given orally. In Expts. 6 and 7 choline chloride (5 mg./rat/day) was given orally, but in later experiments (nos. 9, 10 and 12–15) it was fed with the diet. DL-Methionine (25 mg./rat/day) was also fed with the diet (Expts. 3 and 4).

Food consumption and body weight. Except where otherwise indicated, the food consumption of each animal was measured daily and the body weight on alternate days.

Isolation of serine and glycine

A. *By chromatography of the benzamido acids.* The animals were killed under anaesthesia and the minced viscera (liver, kidney, spleen, reproductive organs, washed gastro-intestinal tract, heart and lungs) of each group which had received the same diet were extracted with ethanol. After two to three extractions with trichloroacetic acid (TCA; 6%, w/v) the tissues (usually 2–4 g.) were again extracted with boiling ethanol and ether and dried at 80°. The protein-rich residue was hydrolysed with 6N-HCl (10 ml./g. tissue) at 100° for 20 hr. Excess HCl was removed by evaporating to dryness *in vacuo* and the amino acids were precipitated with mercuric acetate (Campbell & Work, 1952*a*; cf. Neuberg & Kerb, 1912). The free amino acids were regenerated with H_2S and chromatographed on a Zeo-Karb 225 (Permutit Co. Ltd., London) column (55 × 4 cm. diam., approx. 900 g. wet resin), essentially as described for Dowex 50 resin (Stein & Moore, 1949; Ehrensvärd, Reio, Saluste & Stjernholm, 1951). Fractions containing aspartic and glutamic acids, serine and threonine were combined and the acidic amino acids were removed by adsorption on Amberlite IR-4B resin (Resinous Products Corp., Philadelphia, Pa.). The remaining serine and threonine were converted to the *N*-benzoyl derivatives (Steiger, 1944). The mixture was separated on a column of Hyflo Super-cel (75 g.; Johns Manville Co., London) containing 0·5N-H_2SO_4 (37·5 ml.). The effluent was collected in fractions (30 ml.) which were titrated with 0·02N-KOH in methanol, using cresol red as indicator. With 2% (v/v) *n*-butanol in chloroform there were successively eluted benzoic acid present in the crude mixture of benzamido acids (peak I; fractions 3–6) and *N*-benzoylthreonine (peak II; fractions 9–14); after a further 120 ml. this was followed by 10% (v/v) *n*-butanol in chloroform which eluted *N*-benzoylserine (peak III; fractions 23–27). The fractions containing the potassium salt of *N*-benzoylserine were combined and evaporated to dryness *in vacuo*. The salt was dissolved in water (2–5 ml.), the solution was filtered and acidified with a slight excess of N-HCl. *N*-Benzoylserine crystallized after a few days at 0°. (Yield: approx. 20 mg./g. tissue + equal amount in mother liquors.) Recrystallization from ethyl acetate afforded pure *N*-benzoyl-L-serine, m.p. 148–149° (microblock, uncorrected), of unchanged radioactivity on subsequent recrystallization. *N*-Benzoyl-L-threonine isolated from the appropriate fraction by a similar procedure had m.p. 191°; it was non-radioactive.

The fractions from the Zeo-Karb column containing glycine and alanine were similarly combined and benzoylated. Benzoylalanine and hippuric acid were separated on a Hyflo Super-cel column (75 g.) containing 0·5N-H_2SO_4 (37·5 ml.). Chloroform was used to elute benzoic acid (peak I; fractions 2–3), benzoylalanine (peak II; fractions 4–6) and hippuric acid (peak III; fractions 8–15). Hippuric acid was, however, eluted more conveniently by changing to 10% (v/v) *n*-butanol in chloroform as soon as the benzoylalanine had been washed off the column. All fractions (30 ml.) were titrated with 0·02N-KOH in methanol, appropriate fractions were combined and the hippuric acid was isolated by acidifying the aqueous solution of the potassium salt, as described above; after recrystallization from ethyl acetate:chloroform it had m.p. 190–191° and its radioactivity was unchanged on subsequent recrystallization.

The purity of the benzamido acids was further checked by paper chromatography of the amino acids obtained by hydrolysis with 2N-HCl.

B. *As the dinitrophenyl (DNP) derivative.* In most experiments, glycine was isolated directly from the protein

hydrolysate by treating the mixture with 1-fluoro-2:4-dinitrobenzene and isolating the dinitrophenyl derivative, m.p. 203–205°, after chromatography on Hyflo Super-cel (Perrone, 1951; Campbell & Work, 1952*b*). The radioactivity of this derivative agreed well with that of the hippuric acid isolated by method A.

Degradation of serine

A. *From chromatographically isolated N-benzoylserine.* The following modification of the procedure described by Sakami (1950) was used. *N*-Benzoylserine (0·35 m-mole), isolated as described above, was refluxed with 4 N-HCl (approx. 50 ml.) for 8 hr. The solution was evaporated to dryness and benzoic acid was extracted with light petroleum (b.p. 60–80°). The residue was dissolved in N-NaOH (0·3 ml.) and phosphate buffer (4 ml.; prepared by adding conc. H_3PO_4 to 0·5 M-Na_2HPO_4 to pH = 5·8). The pH was readjusted, if necessary, to 5·8 with 2 N-NaOH, CO_2 was removed by passing CO_2-free N_2 through the solution which was boiled under reflux. To the cooled solution 0·5 M-$NaIO_4$ (2·8 ml.) was added and CO_2-free N_2 was passed through the solution for 2 hr., CO_2 arising from the carboxyl carbon atom of serine being collected as $BaCO_3$. M-$SrCl_2$ (6 ml.) was added and the pH was adjusted to 7 with 2 N-NaOH in order to remove iodate and excess periodate. Formate, derived from the α-carbon atom of serine, was oxidized with $HgCl_2$ to CO_2 and converted into $BaCO_3$ as described by Sakami (1950).

H_2S was passed through the solution, HgS was removed by filtration and the filtrate was concentrated *in vacuo* to approx. 50 ml. Formaldehyde, derived from the β-carbon atom of serine, was isolated as the dimedon derivative by adding dimedon (120 mg.) to the above solution, adjusting the pH to 5 with 2 N-NaOH and warming the mixture to 100° for 15 min. The solution was cooled and the dimedon derivative was filtered off. After recrystallization (aq. ethanol or ethanol) it had m.p. 191°.

B. *Isolation of the β-carbon atom as formaldehyde dimedon from protein hydrolysate.* The mixed viscera were minced and extracted with ethanol and TCA as described above. The dried tissue (1 g.) was hydrolysed with 6 N-HCl for 20 hr. at 100° and the amino acids were purified by precipitation as the Hg complexes, as before (p. 272). After regeneration with H_2S, the amino-acid mixture was oxidized with periodate and the formaldehyde arising from the β-carbon atom of serine was isolated as the dimedon derivative (Rees, 1946).

In one experiment (no. 10, Table 3) the radioactivity of the β-carbon atom of serine obtained by this method was compared with that using method A. The good agreement of the two values confirms the validity of the direct periodate oxidation of the amino-acid mixture for estimating the radioactivity in the β-carbon atom of serine.

RESULTS

Changes of body weight and variations of food consumption with the different diets

Some of the experiments (nos. 6–14, Table 1) had been designed primarily for a study of the biosynthesis of labile methyl groups. For this purpose the cobalamin and choline contents of the diet were varied; the effects of these supplements on growth and food consumption have been discussed in the

Table 1. *Changes of body weight and average food consumption of rats fed various diets containing* [α-^{14}C]*glycine or* L-[β-^{14}C]*serine*

(AAD 1 and AAD 2 are synthetic amino-acid diets (Arnstein & Neuberger, 1953). Two rats were used in each experiment; in Expt. 11 one rat (no. 11*a*) grew exceptionally well, probably owing to intestinal synthesis of cobalamin, whilst the other (no. 11*b*) showed the expected, poor, growth rate for this diet. In this experiment only, the two rats were worked up separately. The diets were supplemented, as indicated, by cobalamin (2·5 μg./rat/day), DL-methionine (25 mg./rat/day) or choline chloride (5 mg./rat/day) designated by the abbreviations B_{12}, Met and Ch respectively.)

Expt. no.	Labelled component fed	Basal diet	Supplement	Duration of experiment (days)	Aggregate body wt. (g.) Initial	Aggregate body wt. (g.) Final	Average food consumption (g./day/rat)
1	2% [α-^{14}C]Glycine (57 μc/mole)	Casein	B_{12}	39	107	266	8·9
2			B_{12}	35	686	716	16·6*
3			None	35	93	189	6·6
4†		AAD 1	Met + B_{12}	21	94	131	4·5
5			Met	23	96	139	4·7
6			B_{12}	23	109	139	4·5
7			Ch + B_{12}	39	108	181	5·7
8			Ch	39	105	90	3·5
9	0·5% [α-^{14}C]Glycine (740 μc/mole)	AAD 2	B_{12}	39	125	171	6·7
10			Ch + B_{12}	39	158	278	9·4
11*a*			Ch	39	79	136	9·9
11*b*			Ch	20	59	68	5·0
12	0·7% L-[β-^{14}C]Serine (220 μc/mole)		B_{12}	20	101	145	5·9
13			Ch + B_{12}	20	134	251	9·1
14			Ch	20	121	143	5·2
15	0·1% [α-^{14}C]Glycine (160 μc/mole)		Ch + B_{12}	21	128	197	8·0
16	0·01% [α-^{14}C]Glycine (160 μc/mole)		Ch + B_{12}	21	128	198	7·9

* Adult rats; daily food consumption was measured during 28 days only.
† The animals in this experiment suffered from a respiratory infection from which one died.

preceding paper (Arnstein & Neuberger, 1953). The growth and food consumption data of the remaining animals (Expts. nos. 1–3, 15–16; Table 1) were of the expected order.

Radioactivities of the glycine and serine isolated from viscera and carcass

In Table 2 the specific radioactivities of the glycine and of the β-carbon atom of serine of the proteins of the viscera and of the carcass respectively are compared with the radioactivities of the corresponding amino acids supplied in the diet. The basal amino-acid diets AAD 1 and AAD 2 contained no glycine or serine, and the labelled amino acids were added to the diets at the concentrations indicated. Casein of cow's milk is reported to have a glycine content of 2 % (Gordon, Semmett & Bender, 1950) and a serine content of 5·9 % (Rees, 1946). In order to allow for the dilution of the labelled glycine by the unlabelled serine and glycine residues in casein, it is assumed that the serine is, on a molar basis, metabolically equivalent to glycine (see Shemin, 1946). The crude casein contained 8 % of ash and water and the corrected casein content of the diet was therefore 11·5 %. The corrected glycine content of the diets used in Expts. 1–3 was calculated to be 2·7 %.

The radioactivity of the glycine isolated from the visceral proteins was remarkably constant in Expts. 4–8 in which diets containing 2 % labelled glycine were used; the values varied between 37 and 44 % of that of the dietary amino acid. There was no apparent effect of cobalamin or choline, or significant variation with the rate of growth. As would be expected, the relative activity of the visceral glycine to that of the fed glycine decreased, as the content of the labelled amino acid in the diet was reduced, being approx. 17 % at the 0·5 % level, 6 % at the 0·1 % level and 0·6 % at the level of 0·01 %. The corrected values with the casein diets (Expts. 1–3; bracketed) were of the order expected for a diet containing about 2·7 % labelled glycine. With 0·7 % [β-^{14}C]serine, the dilution was similar to that found with 0·5 % [α-^{14}C]glycine.

The glycine obtained from the mixed carcass proteins always had a lower specific radioactivity than the glycine isolated from the hydrolysates of the proteins of the viscera. The ratio of the two activities was high and approached unity in experiments in which growth was very good, especially in the two casein experiments in which young rats were used, but in Expt. 8, in which the young rats used lost weight during the experiment, the radioactivity of the carcass glycine was 62 % of that of the glycine of the viscera. By contrast, in the adult rats (Expt. 2) the radioactivity of the carcass glycine remained low, both absolutely and relatively to the visceral glycine, although the

Table 2. *Specific radioactivity of glycine and of the β-carbon atom of serine of the hydrolysed proteins of rats fed* [α-^{14}C]*glycine at various dietary levels or* L-[β-^{14}C]*serine*

(A dietary level of 0·7 % of serine is equivalent on a molar basis to 0·5 % of glycine. Details of the diets, isolation procedures and radioactivity assay are given in the Experimental section; data on growth and food consumption are given in Table 1. The specific radioactivity of the isolated compounds or carbon atoms is expressed as μc/mole or μc/g. atom and has been calculated for a uniform radioactivity, 1 mc/mole, of the dietary precursor. The values in parentheses in columns 5 and 6 are corrected also for the glycine and serine present in the casein fed; for details of the correction see text.)

Expt. no.	Basal diet	Dietary level of labelled compound fed (%)	Supplement*	Specific radioactivity of isolated compounds (μc/mole) or carbon atom (μc/g. atom) Viscera (*a*)	Carcass (*b*)	Ratio 100*b*/*a*
			Glycine experiments			
1	Casein	2	B_{12}	406 (544)	374 (500)	92
2	Casein	2	B_{12}	375 (501)	168 (225)	45
3	Casein	2	None	426 (570)	372 (497)	87
4	AAD 1	2	Met + B_{12}	433	279	64
5	AAD 1	2	Met	433	277	64
6	AAD 1	2	B_{12}	374	258	69
7	AAD 1	2	Ch + B_{12}	440	328	75
8	AAD 1	2	Ch	406	251	62
10	AAD 2	0·5	Ch + B_{12}	157	—	—
11*b*	AAD 2	0·5	Ch	179	—	—
15	AAD 2	0·1	Ch + B_{12}	59·5	34·5	58
16	AAD 2	0·01	Ch + B_{12}	6·5	2·9	45
			Serine experiments			
12	AAD 2	0·7	B_{12}	144	—	—
13	AAD 2	0·7	Ch + B_{12}	153	—	—
14	AAD 2	0·7	Ch	129	—	—

— Signifies value not determined. * For abbreviations used see Table 1.

experiment was extended for 35 days. The low ratio in Expt. 16 is possibly due to errors involved in counting the carcass DNP-glycine sample, which had a very low activity.

Comparing experiments on young rats given similar diets, it is worthy of note that extension of the period of feeding of the labelled compound from 21 or 23 to 39 days does not significantly increase the specific radioactivity of either the visceral or the carcass glycine. The radioactivity found in these experiments for the glycine of the viscera appears to depend entirely on the level of the labelled glycine in the diet and its radioactivity, in contrast to the radioactivity of the glycine of the carcass which depends also on the rate of growth.

The conversion of the α-carbon atom of glycine into the β-carbon atom of serine

Table 3 shows that the extent of this conversion is a function of the glycine content of the diet. The radioactivities of the two carbon atoms of glycine have not been separately determined in this work, but the available evidence, including the distribution of radioactivity in the three carbon atoms of serine shown in Table 3, indicates clearly that feeding α-labelled glycine gives rise only to insignificant radioactivity in the carboxyl group of glycine. It was therefore assumed that the whole of the radioactivity of the glycine was located in the α-carbon atom.

With a glycine content of the diet of 2%, the radioactivity of the β-carbon atom of serine was between 50 and 60% of that of the α-carbon atom of glycine; the corresponding figures for 0·5% dietary glycine were 18–19% and for 0·1% dietary glycine about 11%. On the other hand, the few experiments in which the serine was degraded suggest that the ratio of the radioactivity of the α-carbon atom of glycine to that of the α-carbon atom of serine was not affected by the dietary level of glycine.

DISCUSSION

Quantitative aspects of the endogenous synthesis of glycine and serine

The glycine present in the body proteins of the rats at the end of the experiments is derived from three sources. In the first place, there is the unlabelled glycine which was present in the beginning of the experiments and which has not been replaced. The second fraction comprises the glycine which has been synthesized by the animals during the experiment from various, partly unknown, precursors. This fraction may have a slight radioactivity, since part of the ingested, labelled, glycine is converted into substances which in turn are used for synthesis of new glycine. Thus glycine is converted to some extent into acetate (Sprinson, 1949) and the carbon atoms of the latter are re-utilized for the synthesis of glycine (Arnstein & Neuberger, 1949). However, the dilution factors associated with such reactions are very large and the radioactivity of the glycine fraction which arises by synthesis in the animal can almost certainly be neglected in comparison with the radioactivity of the third fraction, which represents the dietary glycine.

Turnover studies (Shemin & Rittenberg, 1944; Friedberg, Tarver & Greenberg, 1948) indicate that the bulk of the proteins of the viscera of the rat are replaced at rates corresponding to half-life periods

Table 3. *Influence of the level of glycine in the diet on the conversion of the α-carbon atom of glycine into the β-carbon atom of serine*

(All experiments described in this table were done with the mixed proteins of the viscera. Details of the diets and of the isolation, degradation and radioactivity assay are given in the Experimental section and in Table 1. The specific radioactivity of the isolated compounds or carbon atoms is expressed as μc/mole or μc/g. atom and has been calculated for a uniform radioactivity, 1 mc/mole, of the labelled precursor. The values in parentheses in columns 4–6 give the percentage distribution of radioactivity in the three carbon atoms of serine.)

Expt. no.	Dietary level of [α-^{14}C]glycine (%)	Specific radioactivity of isolated compounds or carbon atoms: Glycine (*a*)	Serine: β-C (*b*)	Serine: α-C	Serine: Carboxyl C	Ratio 100*b*/*a*
6	2	374	234	—	—	62
7	2	440	226	—	—	51
8	2	406	239	—	—	59
9	0·5	—	33·6	—	—	—
10	0·5	157	29·7 30·1* (22)	107 (77·5)	0·8 (0·5)	19
11*a*	0·5	—	32·2	—	—	—
11*b*	0·5	179	32·0* (18)	145 (81)	2·0 (1)	18
15	0·1	59·5	6·7* (13)	37·8 (75)	5·7 (11)	11

— Signifies value not determined.
* Value obtained by periodate oxidation of chromatographically pure serine (see Experimental section).

of about 5 days. Thus, after 20 or 39 days about 85 or 99 % respectively of the glycine originally present in the viscera of adult rats should have been replaced by glycine from other sources. In growing animals this 'old' glycine is further diluted owing to a net increase of protein. On the other hand, the mixed proteins of the carcass are replaced more slowly (see Sprinson & Rittenberg, 1949). This is largely due to the relative metabolic inertia of collagen (Neuberger, Perrone & Slack, 1951) which is particularly marked in the adult rat. However, recent experiments (Neuberger & Slack, 1953) have suggested that in the young rat even the glycine of collagen is replaced at an appreciable rate, the turnover rates varying somewhat with different tissues. The results obtained in the present work, involving administration of labelled glycine over relatively long periods, fully support these conclusions which are based on experiments in which the labelled compounds were given over very short periods, and in which the decay of radioactivity was measured. The radioactivity of the glycine obtained from the visceral proteins was relatively constant in all experiments. The lack of effect of the growth rate and of the extension of the feeding period from 20 to 39 days, suggest strongly that almost all the glycine which had been present in the viscera at the beginning of the experiment had been replaced after about 3 weeks. The relative values found for the carcass glycine suggest that only about half of this fraction is replaced during the experimental period and the higher radioactivities found in some experiments can be ascribed to the deposition of new protein associated with growth. The following calculations have therefore been based entirely on the radioactivities of the glycine isolated from the visceral proteins and no correction has been made for 'old' glycine. The error arising from this approximation is likely to be less than 5 % in experiments on animals showing a good growth rate and fed the labelled compound for more than 30 days and to be not more than 10 or at the most 15 % in the less favourable cases.

In order to assess the amounts of glycine synthesized by the rats, the average amount of labelled glycine eaten each day was calculated from the composition of the diet and the observed food consumption (Table 1). The average amount of glycine synthesized (x; in mg./day) was then derived from the equation:

$$x = a(m/n - 1),$$

where a is the average amount of glycine ingested (mg./day) and m and n are the specific radioactivities of the fed and isolated glycine respectively. With the casein diets the values of a and n were corrected for the unlabelled glycine and serine present in the casein. The assumption made that the serine is equivalent to glycine on a molecular basis may not be quite correct but any error thereby introduced is slight. The values thus obtained for different experiments (Table 4) cannot be readily compared owing to differences in final body weights. We have therefore also calculated the average endogenous glycine production y, on a body-weight basis (mg./100 g. body weight/day) with the aid of the following equation:

$$y = 200.x/(\text{initial body weight (g.)} + \text{final body weight (g.)}).$$

In Table 4 the values for y have been expressed in terms of m-moles of glycine. Similar calculations have also been made for serine.

In spite of considerable variations of experimental conditions the values obtained for the daily endogenous glycine synthesis/100 g. body weight are remarkably constant for young rats. The mean value obtained is about 2·5 m-moles/100 g. rat/day, the extreme values being 2·0 and 3·2 m-moles respectively. With the two adult rats the corresponding figure is significantly lower; this may be due to a definite decrease of synthetic capacity with age, but it is probable that the fat content of the bodies of these rats was much higher than that of the younger rats and that the proportion of metabolically active tissue is smaller than the crude body weight would indicate.

Before considering the significance of these findings, it is necessary to examine the validity of an assumption which is implicit in the above calculations. The present treatment assumes that the dietary labelled glycine and the unlabelled glycine synthesized by the rat cannot be distinguished with respect to their metabolic fate. In particular we assume that the probabilities of an ingested amino-acid molecule being irreversibly oxidized or participating in other irreversible or even reversible reactions are equal to those for an amino-acid molecule produced by endogenous synthesis. Assumptions of this type which are made in most metabolic investigations using isotopes are simple and reasonable, provided the amount of labelled substance fed or injected is small. The values for y obtained with diets of a glycine content of 2 and 0·5 % are somewhat higher than those found with diets of glycine content of 0·1 or 0·01 % (Table 4). It is doubtful whether the differences observed, which amount to about 20 %, are statistically significant, but it is possible that the values obtained with the diets containing higher concentrations of glycine are slightly too high. However, the data as a whole, the earlier results on the incorporation of glycine into hippuric acid (Arnstein & Neuberger, 1951) and the observations of Sprinson & Rittenberg (1949) on the rate of ^{15}N excretion after feeding labelled glycine to rats support the assumption that exogenous glycine mixes rapidly with the

Table 4. *Endogenous glycine and serine synthesis calculated from the specific radioactivities of the glycine or serine isolated from the mixed proteins of the viscera and the dietary intake of the labelled amino acid*

(Dietary supplements, initial body weights and average food consumption are given in Table 1; the specific radioactivities of the isolated compounds are given in Table 2. The values in parentheses in column 7 are corrected for the glycine and serine present in casein, making the assumption discussed in the text. The values in the last column are obtained from the following equation: glycine or serine synthesized (mg./day/rat; column 8) × 100/(average body wt. × mol.wt. of glycine or serine) where the average body wt. is obtained by dividing the mean of the aggregate initial and final body weight of each group by the number of animals.)

Expt. no.	Basal diet	Content of diet of labelled glycine or serine (%)	Duration of experiment (days)	Weight change (% of initial)	Average final body wt. (g.)	Labelled amino acid eaten (mg./day/rat)	Calculated amount of glycine or serine synthesized (mg./day/rat)	Calculated amount of glycine or serine synthesized (m-moles/day/100 g. body wt.)
				[α-^{14}C]Glycine; young rats				
1	Casein	2	39	+149	133	178 (238)	198	2·84
3	Casein	2	35	+103	94·5	132 (176)	133	2·52
4	AAD 1	2	21	+39	65·5	90	118	2·79
5	AAD 1	2	23	+45	69·5	94	123	2·79
6	AAD 1	2	39	+28	69·5	90	150	3·24
7	AAD 1	2	39	+72	93·0	114	145	2·63
8	AAD 1	2	39	−14	45·0	70	102	2·80
10	AAD 2	0·5	20	+37	139	47	253	3·09
11*b*	AAD 2	0·5	20	+15	68	25	115	2·41
15	AAD 2	0·1	21	+48	98·5	8	126	2·08
16	AAD 2	0·01	21	+49	99	0·79	121	1·98
				[α-^{14}C]Glycine; adult rats				
2	Casein	2·0	35	+ 4	358	332 (443)	442	1·68
				[β-^{14}C]Serine; young rats				
12	AAD 2	0·7	20	+44	72·5	46·9	281	4·35
13	AAD 2	0·7	20	+95	125·5	63·7	353	3·49
14	AAD 2	0·7	20	+18	71·5	36·1	244	3·52

amino acid already present in the liver and other internal organs. If indeed glycine synthesis occurs exclusively in such tissues, the assumption that mixing is essentially complete before extensive oxidation and other reactions take place would almost certainly be justified. However, a rigorous proof has not yet been produced of the concept that exogenous and endogenous glycine are metabolically equivalent.

The experiments with labelled L-serine (Table 4) were only done at the one dietary level of 0·7%. However, the results indicate clearly that the amounts of serine synthesized by the rat are about 30% greater than those of glycine under comparable conditions. This and the finding that, even after prolonged feeding of labelled glycine, the isotope content of the body glycine exceeds that of the serine by 20–35% suggests that it is primarily serine which is synthesized from nitrogen-free precursors and that most of the synthetic glycine is derived from serine. This amino acid is required for several reactions not involving glycine, such as the formation of ethanolamine and cystathionine, and it is therefore to be expected that its rate of synthesis will exceed that of glycine, if it is to function as the main precursor of the latter.

It follows from the data in Table 4 that the rate of endogenous synthesis of glycine and presumably also that of serine is independent of the glycine or serine content of the diet. The exact amounts of glycine and serine required by the young rat for maximum growth (about 4 g./day) cannot be estimated with great accuracy. The mixed proteins of the rat contain about 10% of glycine (Dunn, Camien, Malin, Murphy & Reiner, 1949) and approx. 5% of serine (Sauberlich & Baumann, 1951); for the synthesis of 700–800 mg. of protein/day about 100–110 mg. of glycine and serine are thus needed. If allowance is made for the glycine and serine required for the formation of nucleic acids, porphyrin, ethanolamine, cystine, choline and creatine, the amounts of these amino acids necessary for maximum growth may be estimated to be about 1·5–2·0 m-moles/day. This figure is of the same order of magnitude as that found for endogenous glycine production in the present work. It would thus appear that the quantities of the two amino acids formed by endogenous reactions do not greatly exceed those needed for maximum growth. The effect of benzoate on growth indicates that the removal of glycine by conjugation does not stimulate an increase in the rate of synthesis of this

amino acid. Conversely, an increased supply of glycine in the diet does not lead to a reduction of synthesis.

The values obtained here include both the glycine synthesized from nitrogen-free precursors, probably through serine, and that produced specifically from threonine (Meltzer & Sprinson, 1952). However, the proportion of glycine which can arise from dietary threonine is relatively small. None of the many other amino acids investigated has been found to be an efficient glycine precursor (Åqvist, 1951).

The present experiments do not suggest that cobalamin is concerned with the synthesis of glycine or, particularly, the conversion of serine into glycine, since the rate of endogenous glycine formation is not significantly affected by the presence of cobalamin in the diet. In this respect cobalamin differs from folic acid which has been reported to be involved in glycine formation from serine (Elwyn & Sprinson, 1950). It is possible, however, that none of the rats used in the present experiments was completely deficient in cobalamin, although its absence from the diet affected growth and food consumption under the conditions used.

The conversion of the α-carbon atom of glycine into the β-carbon of serine

It has been known for some time that in the rat the nitrogen and the α- and carboxyl-carbon atoms of serine are in rapid equilibrium with the corresponding atoms of glycine. In the conversion of serine into glycine the β-carbon atom is lost (Shemin, 1946) and in the reverse reaction glycine condenses with a one-carbon fragment, which can originate from labile methyl groups (Sakami, 1949*b*; Siekevitz & Greenberg, 1950), acetone (Sakami 1950), the β-carbon atom of serine, formaldehyde (Siegel & Lafaye, 1950), formate (Sakami, 1948), as well as the α-carbon atom of glycine (Sakami, 1949*a*; Siekevitz & Greenberg, 1949). In the early experiments on the conversion of glycine into serine (Sakami, 1949*a*) rats were fed doses of 5 m-moles [α-^{14}C]glycine/100 g. body weight. Under these conditions, the radioactivity of the β-carbon atom of serine was 82 % of that of the α-carbon atom. Later, Weissbach, Elwyn & Sprinson (1950), investigating the biosynthesis of choline, showed that after feeding [α-^{14}C]glycine (0·4 m-mole/100 g. body weight) the carbinol carbon atom of the ethanolamine moiety contained about 24 % of the radioactivity of the amine carbon atom. Since the ethanolamine moiety of choline is known to be synthesized in the rat by decarboxylation of serine (Arnstein, 1951), it seems likely that the distribution of radioactivity in the β- and α-carbon atoms of serine was similar to that in the corresponding carbon atoms of choline. The above evidence suggested that the extent of the conversion of the α-carbon atom of glycine into the β-carbon atom of serine might be dependent on the amount of glycine fed, although the experimental conditions in these experiments were not strictly comparable and the observed differences might therefore have been due to other factors.

As it is still uncertain which of the possible precursors of the β-carbon atom of serine is quantitatively the most important, it was of interest to determine the extent to which this carbon atom is derived from the α-carbon atom of glycine under normal conditions. The experiments in Table 3 show clearly that this reaction is markedly affected by the level of glycine in the diet. Although the serine in Expts. 6–8 was not degraded, the radioactivity of the serine α-carbon atom could not have exceeded that of the glycine and it may therefore be calculated that the radioactivity of the β-carbon atom of serine was at least 60 % of that of the α-carbon atom. In these experiments glycine was fed at the rate of about 1·8 m-moles/100 g. body weight/day. When the level of glycine in the diet was reduced to 0·5 m-mole (Expts. 10 and 11*b*) and 0·1 m-mole/100 g. body weight/day (Expt. 15) the corresponding radioactivity in the β-carbon atom of serine was about 22 and 17 % of that in the α-carbon atom of glycine. Since reduction of the dietary level of glycine did not increase the rate of endogenous glycine synthesis (see above), the relative decrease in the radioactivity of the β-carbon atom of serine on the low glycine diets must be due to a reduction in the conversion of the α-carbon atom of glycine into the β-carbon atom of serine. It may be deduced from this that endogenous glycine is not an important source of the β-carbon atom of serine and that this conversion, involving formaldehyde or formate as likely intermediates, is mainly used to deal with excessive amounts of dietary glycine.

The effect of the level of a compound in the diet on the rate of its metabolism, similar to that observed in this work, has been noted previously. Thus it has been shown (Mackenzie, Rachele, Cross, Chandler & Du Vigneaud, 1950) that an increase in the amount of L-methionine in the diet from 0·6 to 1·2 % resulted in a ninefold increase in the oxidation of the methyl group to carbon dioxide. Similarly, the rate of transfer of the methyl group of methionine to creatine was also not proportional to the level of methionine in the diet (Cohn, Simmonds, Chandler & Du Vigneaud, 1946).

It has been realized for some time (see e.g. Moss & Schoenheimer, 1940) that interconversion of two compounds occurs in animals, even when adequate amounts of both substances are supplied in the diet. However, most of these observations have been of a qualitative character. The present results on glycine synthesis show clearly that the daily quantity of this amino acid formed by the rat is independent of the amounts supplied in the diet.

On the other hand, the rate of conversion of the α-carbon atom of glycine into the β-carbon atom of serine increases with the glycine content of the diet. It would thus follow that the relatively constant level of glycine in the tissues is maintained mainly by regulation of the rate of disposal of glycine rather than of the rate of formation.

SUMMARY

1. Amino acid or casein diets containing 0·01, 0·1, 0·5 or 2 % [α-^{14}C]glycine or 0·7 % L-[β-^{14}C]serine were fed to groups of rats over periods extending from 20 to 39 days. The glycine was isolated from the proteins of the mixed viscera and of the carcass. The serine was isolated and degraded in some experiments whilst in others the radioactivity of the β-carbon atom was estimated after periodate oxidation without isolation. Food consumption and changes of body weight were measured.

2. Using diets containing 2 % [α-^{14}C]glycine it was shown that the specific radioactivity of the glycine of the visceral proteins, which was about 40 % of that of the fed amino acid, was not affected by the rate of growth nor by variation of the feeding period within the limits mentioned. On the other hand, the radioactivity of the glycine of the carcass increased with increasing growth rate.

3. It was assumed that the amino acid present in the visceral protein at the beginning of the experiment was replaced almost completely and that the dietary glycine or serine and the glycine or serine synthesized by the animal are metabolically indistinguishable. The average daily amount of glycine or serine synthesized was calculated from the food consumption and the radioactivities of the fed and isolated amino acid.

4. The values for glycine synthesis were constant, independent of the level of glycine in the diet, the mean being 2·5 m-moles/100 g. body weight/day. The corresponding figure for serine was 3·5, suggesting that endogenous glycine is largely derived from serine.

5. The conversion of the α-carbon atom of glycine into the β-carbon atom of serine was greatly diminished when the level of glycine in the diet was decreased, indicating that this pathway may be concerned with the disposal of excess glycine.

6. It is concluded that the constant level of glycine in the tissues is maintained by regulation of the rate of degradation and not by variations in the rate of synthesis.

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REFERENCES

Almquist, H. J. & Grau, C. R. (1944). *J. Nutr.* **28**, 325.
Almquist, H. J., Stokstad, E. L. R., Mecchi, E. & Manning, P. D. V. (1940). *J. biol. Chem.* **134**, 213.
Åqvist, S. E. G. (1951). *Acta. chem. scand.* **5**, 1046.
Arnstein, H. R. V. (1951). *Biochem. J.* **48**, 27.
Arnstein, H. R. V. & Neuberger, A. (1949). *Biochem. J.* **45**, iii.
Arnstein, H. R. V. & Neuberger, A. (1951). *Biochem. J.* **50**, 154.
Arnstein, H. R. V. & Neuberger, A. (1953). *Biochem. J.* **55**, 259.
Campbell, P. N. & Work, T. S. (1952*a*). *Biochem. J.* **50**, 449.
Campbell, P. N. & Work, T. S. (1952*b*). *Biochem. J.* **52**, 217.
Cohn, M., Simmonds, S., Chandler, J. P. & Du Vigneaud, V. (1946). *J. biol. Chem.* **162**, 343.
Csonka, F. A. (1924). *J. biol. Chem.* **60**, 545.
Dunn, M. S., Camien, M. N., Malin, R. B., Murphy, E. A. & Reiner, P. J. (1949). *Univ. Calif. Publ. Physiol.* **8**, 293.
Ehrensvärd, G., Reio, L., Saluste, E. & Stjernholm, R. (1951). *J. biol. Chem.* **189**, 93.
Elwyn, D. & Sprinson, D. B. (1950). *J. biol. Chem.* **184**, 475.
Friedberg, F., Tarver, H. & Greenberg, D. M. (1948). *J. biol. Chem.* **173**, 355.
Gordon, W. G., Semmett, W. F. & Bender, M. (1950). *J. Amer. chem. Soc.* **72**, 4282.
Griffith, W. H. (1929). *J. biol. Chem.* **82**, 415.
Griffith, W. H. & Lewis, H. B. (1923). *J. biol. Chem.* **57**, 1.
Jukes, T. H., Stokstad, E. L. R. & Belt, M. (1947). *J. Nutr.* **33**, 1.
Kratzer, F. H. & Williams, D. (1948). *J. Nutr.* **35**, 315.
McCoy, R. H. & Rose, W. C. (1937). *J. biol. Chem.* **117**, 581.
Mackenzie, C. G., Rachele, J. R., Cross, N., Chandler, J. P. & Du Vigneaud, V. (1950). *J. biol. Chem.* **183**, 617.
Magnus-Levy, A. (1907). *Biochem. Z.* **6**, 523.
Meltzer, H. L. & Sprinson, D. B. (1952). *J. biol. Chem.* **197**, 461.
Moss, A. R. & Schoenheimer, R. (1940). *J. biol. Chem.* **135**, 415.
Neuberg, C. & Kerb, J. (1912). *Biochem. Z.* **40**, 498.
Neuberger, A., Perrone, J. C. & Slack, H. G. B. (1951). *Biochem. J.* **49**, 199.
Neuberger, A. & Slack, H. G. B. (1953). *Biochem. J.* **53**, 47.
Perrone, J. C. (1951). *Nature, Lond.*, **167**, 513.
Rees, M. W. (1946). *Biochem. J.* **40**, 632.
Rose, W. C., Burr, W. W. & Sallach, H. J. (1952). *J. biol. Chem.* **194**, 321.
Sakami, W. (1948). *J. biol. Chem.* **176**, 995.
Sakami, W. (1949*a*). *J. biol. Chem.* **178**, 519.
Sakami, W. (1949*b*). *J. biol. Chem.* **179**, 495.
Sakami, W. (1950). *J. biol. Chem.* **187**, 369.
Sauberlich, H. E. & Baumann, C. A. (1951). *Cancer Res.* **11**, 67.
Shemin, D. (1946). *J. biol. Chem.* **162**, 297.

Shemin, D. & Rittenberg, D. (1944). *J. biol. Chem.* **153**, 401.
Siegel, I. & Lafaye, J. (1950). *Proc. Soc. exp. Biol., N.Y.*, **74**, 620.
Siekevitz, P. & Greenberg, D. M. (1949). *J. biol. Chem.* **180**, 845.
Siekevitz, P. & Greenberg, D. M. (1950). *J. biol. Chem.* **186**, 275.
Sprinson, D. B. (1949). *J. biol. Chem.* **178**, 529.
Sprinson, D. B. & Rittenberg, D. (1949). *J. biol. Chem.* **180**, 715.
Steiger, R. E. (1944). *J. org. Chem.* **9**, 396.
Stein, W. H. & Moore, S. (1949). *Cold Spr. Harb. Symp. quant. Biol.* **14**, 179.
Weissbach, A., Elwyn, D. & Sprinson, D. B. (1950). *J. Amer. chem. Soc.* **72**, 3316.
White, A. (1941). *Yale J. Biol. Med.* **13**, 759.
Wiechowski, W. (1906). *Beitr. chem. Physiol. Path.* **7**, 204.

The Metabolism of DL-[β-^{14}C] and DL-[^{35}S]Cystine by the Rat

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The two amino acids, serine and cystine (or cysteine), show a striking resemblance in some aspects of their intermediary metabolism. They are quantitatively similar in their glycogenic action (Dakin, 1913) and have been shown to be metabolized to pyruvate: cysteine and cystine by liver extracts (Smythe, 1942) and serine by liver extracts (Chargaff & Sprinson, 1943*a*), bacterial suspensions (Gale & Stephenson, 1938; Chargaff & Sprinson, 1943*b*) and probably also *in vivo* by the rat (Arnstein, 1951*a*). Furthermore, the aminoethanethiol moiety of coenzyme A (Lynen, Reichert & Rueff, 1951) may be derived from cysteine by decarboxylation. Such a reaction would be analogous to the biosynthesis of the ethanolamine moiety of choline from serine by the rat (Stetten, 1942; Arnstein, 1951*b*).

It was therefore thought that the β-carbon atom of cystine might be converted in the rat to a one-carbon compound by a reaction similar to that occurring in the formation of glycine from serine, and the present investigation deals with the metabolism of DL-[β-^{14}C]cystine and its possible conversion to a one-carbon precursor of choline and methionine methyl groups. At the same time, a preliminary experiment was carried out with DL-[γ-^{14}C]valine, which seemed another possible methyl-group precursor, but neither amino acid was found to contribute significantly to the biosynthesis of methyl groups. The conversion of cystine to certain other amino acids and the possible incorporation of cystine sulphur into methionine was also investigated in the course of this work.

EXPERIMENTAL

Animals and diet. The animals used in these experiments were albino rats of Institute stock. They were kept on the stock diet previously described (Arnstein, 1951*b*). The isotopically labelled compounds were fed overnight mixed with diet (10 g./rat) containing D-glucose (0·5 g.).

Valine experiment. Two male rats (aggregate body wt. = 479 g.), which had been fasted for 24 hr., were fed DL-[γ-^{14}C]valine (11·7 mg., 16·7 μc) together with unlabelled L-valine (107·0 mg.) mixed with the diet which also contained α-amino-γ-phenylbutyric acid (200 mg.) and *p*-aminobenzoic acid (200 mg.).

Cystine experiments. DL-[β-^{14}C]Cystine (33 mg., 20·8 μc) was fed to two female rats (aggregate body wt. = 512 g.) previously fasted for 24 hr., together with unlabelled L-cystine (50 mg.) in the diet which also contained α-amino-γ-phenylbutyric acid (200 mg.) and *p*-aminobenzoic acid (200 mg.). DL-[^{35}S]Cystine (3·7 mg., 110 μc) was fed to one female rat (body wt. = 215 g.), together with unlabelled L-cystine (25 mg.).

Measurements of radioactivity. The specific radioactivities of all compounds were determined with a bell-shaped helium-filled Geiger-Müller counter having a thin mica window. The measurements were made on solid samples of 'infinite thickness' mounted on polythene disks (Popják, 1950) and compared with a standard consisting of ^{14}C-labelled polymethyl methacrylate supplied by the Radiochemical Centre, Amersham.

Methods of isolation

Hippuric and acetamido acids from the urine. The isolation of hippuric acid and L-α-acetamido-γ-phenylbutyric acid and *p*-acetamidobenzoic acid from the urine was carried out as previously described (Arnstein, 1951*a*).

Choline. Choline was isolated from the carcass and the viscera as the reineckate, which was converted to the chloroplatinate (Du Vigneaud, Cohn, Chandler, Schenck & Simmonds, 1941) for radioactivity assay.

Amino acids from the visceral proteins of rats which had been fed DL-[β-^{14}C]*cystine.* The rats were killed under anaesthesia about 44 hr. after they had been given the food containing the labelled compound. The viscera (liver, kidney, spleen, heart, lungs and gonads) were comminuted and extracted several times with 95% (v/v) ethanol to remove lipids. The residue was extracted three times with 5% (w/v) trichloroacetic acid (TCA), washed with ethanol and ether and dried at 80°. The dried protein (4·5 g.) was hydrolysed with 6N-HCl (45 ml.) at 100° for 24 hr. After removal of the HCl by evaporation the amino acids were purified by precipita-

tion as the Hg complexes (Campbell & Work, 1952; Neuberg & Kerb, 1912). The mixture was regenerated by H_2S and chromatographed on a column (50 cm. × 4·5 cm. diam.) of Zeo-Karb 225 resin, using 1·5 N-, 2·5 N- and 4 N-HCl successively as eluants, essentially as described for Dowex 50 resin (Stein & Moore, 1950; Ehrensvärd, Reio, Saluste & Stjernholm, 1951), and individual amino acids were isolated as described below.

Aspartic and glutamic acids were isolated by evaporating the appropriate fractions to dryness and recrystallizing samples of the residue from water until their radioactivity remained unchanged.

Serine, threonine, glycine and alanine were isolated as the *N*-benzoyl derivatives, which were separated chromatographically and recrystallized to constant radioactivity (Arnstein & Neuberger, 1953*a*).

Valine and leucine were obtained as the hydrochlorides by evaporating to dryness the fractions containing these amino acids. The crude solids were assayed directly for ^{14}C without further purification. Fractions containing leucine + methionine were similarly assayed and as the radioactivities of these fractions were very low these amino acids were not further purified.

Cystine was isolated by evaporating the appropriate fractions to dryness, dissolving the residue in water and adjusting the pH of the filtered solution to about 5 by adding pyridine. The material which separated was filtered off on to filter paper mounted on a perforated disk (Popják, 1950) and assayed for ^{14}C after drying. Owing to the small amount of material available further purification was not practicable.

The separation of the amino acids was checked by paper chromatography of the isolated compounds.

Assay of ^{35}S from methionine and cystine of the visceral protein of a rat which had received DL-[^{35}S]*cystine*

In this experiment the animal was killed approx. 36 hr. after being fed the labelled amino acid. The viscera were comminuted, extracted with ethanol and TCA and hydrolysed as before. The amino acids were again purified by precipitation as the Hg complexes and regenerated with H_2S. The amino-acid mixture thus obtained from 200 mg. protein was chromatographed on paper (Whatman no. 3, 22·5 × 18·25 in.). The mixture was applied in solution as evenly as possible along the base line (31 cm.) and methionine and cystine markers were run on either side of the mixture. The chromatogram was eluted (18 hr.) with phenol-water (5:2, v/v) in the presence of NH_3. The position of the markers was detected by spraying the ends of the paper with ninhydrin. Methionine was located at 25–29 cm. from the base line, cystine at 7–13 cm., the solvent front being at 30 cm. Methionine and cystine were then eluted from the appropriate central portions of the paper with water and 2 N-HCl respectively. The methionine fraction was rechromatographed on another paper, using amyl alcohol: pyridine: water (3·5:3·5:3, v/v/v) as eluant. The methionine was eluted from the appropriate section of the paper with water, and the solution was evaporated to dryness. A two-dimensional chromatogram (amyl alcohol: pyridine and phenol: water/NH_3, as before) showed that this material contained no cystine, although amino acids other than methionine were still present. The methionine sulphur was therefore converted to sulphate by digesting the mixture with Pirie's reagent (Pirie, 1932). The sulphate was isolated as benzidine sulphate, which was assayed for ^{35}S on a filter paper mounted on a 1 cm.^{2} perforated metal disk, the amount of material assayed being estimated subsequently by titration with 0·01 N-NaOH (cf. Simpson & Tarver, 1950).

The cystine fraction was treated with Br_2 water and the mixture was rechromatographed on paper using phenol-water/NH_3 as before. The cysteic acid, which was well separated from the other amino acids, was eluted with water, converted to sulphate and assayed as benzidine sulphate as described above.

Degradation of the isolated amino acids and of the acetic acid from the acetamido acids

Serine. This amino acid was degraded by periodate oxidation, using the modification of the procedure of Sakami (1950) described earlier (Arnstein & Neuberger, 1953*a*).

Alanine. Benzoylalanine (0·5 m-mole) which had been isolated chromatographically, was hydrolysed by refluxing with 4 N-HCl (25 ml.) for 6 hr. The solution was evaporated to dryness and the solid was extracted with ether. The residue was dissolved in 0·5 M phosphate buffer (pH 5·8), the solution was refluxed in a stream of CO_2-free N_2 to expel CO_2, ninhydrin (0·5 g.) was added and the solution was again refluxed. Acetaldehyde was trapped in 50% (v/v) aqueous methanol cooled in freezing mixture (−10 to −15°) and CO_2 was absorbed in $Ba(OH)_2$. The acetaldehyde was immediately further degraded to iodoform by adding 2 N-NaOH (10 ml.) followed by a mixture of iodine (0·5 g.) KI (0·5 g.), 2 N-NaOH (10 ml.) and 50% (v/v) aq. methanol (20 ml.).

Glutamic acid. Glutamic acid (85 mg.) was dissolved in water (5 ml.) and after addition of 0·5 M phosphate buffer (pH 5·8) the solution was refluxed in a stream of CO_2-free N_2. Ninhydrin (0·3 g.) was then added, the solution was again refluxed and the CO_2, which was derived from the α-carboxyl group, was collected as $BaCO_3$.

Acetic acid. Silver acetate, obtained by hydrolysis of α-acetamido-γ-phenylbutyric acid or *p*-acetamidobenzoic acid, was degraded by decarboxylation with Br_2 in dry CCl_4 (Arnstein, 1951*a*).

Synthesis of labelled compounds

DL-[γ-^{14}C]*Valine.* This amino acid was obtained from the Radiochemical Centre, Amersham.

DL-[^{35}S]*Cystine.* DL-[^{35}S]Cystine was synthesized by Mr P. T. Grant by condensation of [^{35}S]thioacetic acid (ethanethiolic acid), obtained from the Radiochemical Centre, with α-acetamidoacrylic acid; the product, *N*-[^{35}S]-diacetylcysteine, was hydrolysed and converted to DL-[^{35}S]cystine by oxidation with air.

DL-[β-^{14}C]*Cystine.* The synthesis of this compound is based on the method described by Crawhall & Elliott (1951). In the modification described here, no transfer of radioactive material is required until the last stage. $^{14}CH_3OH$ (approx. 150 mg., 0·9 mc) was converted to an aqueous solution of $^{14}CH_2O$ (approx. 104 mg./5 ml.) (Arnstein, 1951*a*), which was added to a 10% excess of ethyl *N*-benzylthiothiocarbonylaminomalonate (1·30 g.) in pyridine (10 ml.). The mixture was kept at room temp. overnight, 10 mg. unlabelled formaldehyde (in dil. aq. solution) was added, and after a further 7 hr. the solvent was evaporated *in vacuo* at <60°. The residue was dried *in vacuo* over conc. H_2SO_4 overnight. Anhydrous ether (5 ml.) was added to the viscous oil and the solution was cooled in an ice-salt bath.

Purified thionyl chloride (10 ml.) (Martin & Fieser, 1943), also previously cooled, was added dropwise to the ether solution at a slow rate. The mixture was allowed to stand in the cold for 1 hr. after the addition of the thionyl chloride; as much of the thionyl chloride as possible was then removed by evaporation *in vacuo* at <20°. 5N-NaOH in ethanol was then added to the residual oil till the solution was alkaline to litmus. After 20 min. the alkaline solution was neutralized with 3N-HCl and the ethanol removed under reduced pressure. Excess 3N-HCl was added and the solution was refluxed for 2 hr. After removal of the HCl under reduced pressure, water (25 ml.) followed by N-NaOH (25 ml.) was added to give a strongly alkaline solution which was allowed to stand for 30 min. with occasional stirring. After addition of conc. HCl (50 ml.) the solution was refluxed for 24 hr. The HCl and some benzoyl mercaptan were removed by evaporation *in vacuo*, water being added to assist the removal of the last traces. Water (50 ml.) was added and the solution was twice extracted with ether, the ether layer being removed with a pipette. After evaporation of the aqueous layer, the residue was extracted with ethanol (10 ml.). The insoluble material was filtered off and washed with more ethanol. The filtrate (40 ml.) was diluted with an equal volume of water and the solution was oxidized with 0·2N-iodine (required: 10 ml., 52% yield) using starch as an external indicator. Excess iodine was removed with a drop of sodium thiosulphate solution and the pH of the solution was adjusted to 5 by adding ammonia (sp.gr. 0·880) till alkaline, followed by acetic acid.

After 20 hr. at 0° the [β-^{14}C]cystine was filtered off and dried (yield: 0·2193 g., 47·5% based on ethyl *N*-benzylthiothiocarbonylaminomalonate; specific radioactivity = 0·63 μc/mg.). The radioactivity of this material was unchanged after recrystallization from water.

RESULTS

Conversion of cystine and valine to choline. The choline isolated from the carcass and viscera from the rats which had been fed DL-[β-^{14}C]cystine had a specific radioactivity of 0·24 μc/mole; that isolated after feeding DL-[γ-^{14}C]valine was completely non-radioactive.

Comparison of the conversion of cystine and valine to 'acetate'. The incorporation of radioactivity into the acetyl derivative of α-amino-γ-phenylbutyric acid (Table 1) shows that DL-[β-^{14}C]cystine and DL-[γ-^{14}C]valine are converted by the rat to 'acetate'. In the valine experiments the acetyl derivative of *p*-aminobenzoic acid was also radioactive. The dilution factors for the conversion of both amino acids and dietary acetate to α-acetamido-γ-phenylbutyric acid are of the same order of magnitude. Degradation of the acetic acid obtained by hydrolysis of the α-acetamido-γ-phenylbutyric acid excreted by animals which had been fed DL-[β-^{14}C]cystine indicated that 95% of the radioactivity was located in the methyl carbon atom. This distribution of isotope is very similar to that found in the acetyl carbon atoms of α-acetamido-γ-phenylbutyric acid after feeding $^{14}CH_3CO_2Na$ when 98–99% of the ^{14}C was located in the methyl carbon atom (Arnstein, 1951*b*).

Conversion of cystine to glycine. The radioactivity of the hippuric acid excreted after feeding DL-[β-^{14}C]cystine (Table 1) was very much lower than that of the α-acetamido-γ-phenylbutyric acid, indicating that [β-^{14}C]cystine is converted to glycine only to a small extent, which is also shown by the correspondingly high dilution factor. Similarly, the radioactivity of the glycine isolated from the protein of the mixed viscera (Table 2) was low, being only about 0·7% of that of the cystine from the same source.

Table 1. *Specific radioactivities of compounds isolated from the urine of rats which had received* DL-[β-^{14}C]*cystine*, DL-[γ-^{14}C]*valine or sodium* [α-^{14}C]*acetate in the diet*

(Details of the experiments are given in the Experimental section. The experiment with labelled acetate has been described previously (Arnstein, 1951*b*) and is included for comparison. The values in brackets in columns 4–6 are the dilution factors, which have been calculated from the radioactivities of the fed and isolated compounds (Arnstein, 1951*a*). The dilution factor represents the dilution of isotope under standard conditions, i.e. it is the dilution which would have been observed if exactly 1 m-mole of labelled compound/100 g. body weight had been fed.)

Details of labelled compound fed			Specific radioactivity of isolated compounds (μc/mole) and dilution factors of isotopic tracer (in brackets)		
Compound	Specific radioactivity (μc/m-mole)	Amount given (m-mole/100 g. body wt.)	Hippuric acid	*p*-Acetamido-benzoic acid	L-α-Acetamido-γ-phenylbutyric acid
DL-S-^{14}CH$_2$.CHNH$_2$.CO$_2$H / S-^{14}CH$_2$.CHNH$_2$.CO$_2$H	31·6*	0·13*	7·1 (580)	—	112† (36·0)
DL-^{14}CH$_3$ \ CH.CHNH$_2$.CO$_2$H / ^{14}CH$_3$	16·7	0·21	—	281 (13·1)	186 (19·8)
^{14}CH$_3$CO$_2$Na	1000	0·0069	14·1 (490)	—	376 (18·4)

—, Value not determined.

* Calc. as cysteine.

† Degradation of the acetic acid obtained by hydrolysis showed that 95% of the radioactivity was located in the methyl-carbon atom of the acetate.

Conversion of cystine to other amino acids. In Table 2 the specific radioactivity of cystine isolated from the visceral protein of rats fed DL-[β-^{14}C]-cystine is compared with the radioactivities of glutamic and aspartic acids, alanine, serine, leucine, valine and threonine. The radioactivity of a mixture of leucine and methionine was also determined and found to be equal to that of the leucine fraction, indicating that the radioactivity of the methionine is approximately the same as that of the leucine.

Table 2. *Specific radioactivities of amino acids isolated from the protein of the mixed viscera of rats which had been fed* DL-[β-^{14}C]*cystine*

(Details concerning the administration of the labelled cystine and the isolation, degradation and radioactivity assay of the amino acids are given in the Experimental section. For comparison, the specific radioactivity is expressed as μc/mole or μc/g. atom. The cystine fed had a radioactivity of 30 μc/m-mole cysteine.)

Amino acid	Specific radioactivity (μc/mole or μc/g. atom)	Radioactivity in individual carbon atoms (%)
Cystine	224·4*	—
Glutamic acid:		
Total	26·0	—
α-Carboxyl-carbon atom	3·4	13
Alanine:		
Total	22·4	—
β-Carbon atom	15·8	71
α-Carbon atom	5·6†	25
Carboxyl-carbon atom	1·0	4
Aspartic acid	16·4	—
Serine:		
Total	5·2	—
β-Carbon atom	3·4	60
α-Carbon atom	1·5	26
Carboxyl-carbon atom	0·8	14
Glycine	1·6	—
Methionine + leucine	1‡	—
Leucine	1‡	—
Valine	0·5‡	—
Threonine	<0·06	—

* Calc. as cysteine.
† Calc. by difference.
‡ Approx. values, based on radioactivity of crude amino-acid fractions (see Experimental section).

The specific radioactivity of the cystine isolated from the visceral protein was only about 0·8 % of that of the fed amino acid. Glutamic acid had about 0·1 % of the specific radioactivity of the fed cystine, which is higher than that of the other amino acids isolated with the exception of cystine itself. Aspartic acid and alanine contained almost as much radioactivity as the glutamic acid, but serine was much less radioactive. Glycine contained approximately as much ^{14}C as the α- and carboxyl-carbon atoms of serine, which are known to be in equilibrium with glycine (see, for example, Arnstein & Neuberger, 1953*a*). Methionine, leucine and valine appear to be weakly radioactive, but these amino acids were not isolated in a pure state and it is possible that their activity was due to small amounts of impurities. The radioactivity of threonine was too low to be measured accurately and it seems likely that this amino acid was in fact non-radioactive.

Table 3. *Specific radioactivity of the sulphur of cystine and methionine from the mixed visceral proteins of a rat which had been fed* DL-[^{35}S]*cystine*

(Details of the administration of the labelled cystine and the isolation methods are given in the Experimental section. The radioactivity of the sulphur was determined after conversion to benzidine sulphate, which was estimated by titration with 0·01 N-NaOH. Using this value, the observed counts/min. were converted to counts/min./mg. S. In order to facilitate comparison with the fed cystine, the radioactivity is also expressed as μc/m-mole, which was obtained by comparing the observed counting rate with that of a standard of known radioactivity. The term 'dilution factor' is defined in Table 1. The value in brackets in column 4 is the dilution factor for the cystine isolated from the visceral protein of rats fed DL-[β-^{14}C]-cystine (Tables 1 and 2) and has been included for comparison.)

Compound	Specific radioactivity: Counts/min./mg. S	Specific radioactivity: μc/m-mole	Dilution factor
Cystine	12 050	2·06	39 (19)
Methionine	80	0·014	5500

Table 3 shows that after feeding [^{35}S]cystine the methionine of the visceral protein has only 0·7 % of the specific radioactivity of the cystine from the same source. It appears therefore that cystine sulphur is not incorporated into methionine to any significant extent.

DISCUSSION

Recent experiments on the synthesis of the methyl groups of methionine and choline by rats fed diets deficient in or devoid of labile methyl groups have shown that the β-carbon atom of serine is an important methyl-group precursor, which can give rise to about 70 % of the newly synthesized methyl groups (Arnstein & Neuberger, 1953*b*). It seemed likely that the remaining 30 % of the newly synthesized methyl groups are derived from other constituents of the diet, although the possibility that methyl groups already present in the body at the beginning of the experiment were re-utilized to some extent, could not be excluded. It is known that histidine (Reid & Landefeld, 1951; Soucy & Bouthillier, 1951; Toporek, Miller & Bale, 1952) and tryptophan (Knox & Mehler, 1950) can give rise to one-carbon fragments which are subsequently converted to methyl groups. Cysteine appeared to be another possible methyl-group precursor if the

β-carbon atom of this amino acid were split off to a one-carbon compound by a similar reaction to that occurring during the conversion of serine to glycine. Alternatively, reversal of the formation of cysteine from methionine (or homocysteine) and serine and cleavage of the intermediate, cystathionine, into glycine and a homocystine derivative, such as *S*-formylhomocysteine, might lead to the formation of a one-carbon fragment from the β-carbon atom of cysteine. Such a reaction would not be expected to be a major pathway of cysteine metabolism as it is known from experiments with rats that cysteine cannot replace methionine nutritionally, even in the presence of choline and homoserine (Armstrong & Binkley, 1948) or α-aminobutyric acid (Fromageot & Clauser, 1949), which might serve as sources of methyl groups and the carbon chain of homocysteine respectively. Furthermore, L-cystathionine, fed together with choline or cobalamin, also does not support the growth of rats on a diet lacking methionine (Stekol & Weiss, 1950; Anslow, Simmonds & Du Vigneaud, 1946), although homocysteine is effective under these conditions. This indicates that the sulphur of cysteine or cystathionine is not available for the synthesis of a sufficient amount of methionine. On the other hand, L-*allo*cystathionine appears to be effective in replacing methionine to a small but definite extent (Anslow *et al.* 1946), and it seemed possible, therefore, that the results of the above nutritional studies might be due to an insufficiently fast conversion of cysteine to methionine rather than to a complete absence of such a reaction. This possibility was investigated in the present work with [^{35}S]cystine. It was found that cystine sulphur is incorporated into methionine only to a very small extent, which may indicate a low degree of reversibility of the conversion of methionine sulphur into cystine. However, methionine was not isolated in this experiment and it is therefore possible that the observed radioactivity in the methionine fraction was due to an impurity, e.g. a cysteine peptide, which was not completely separated.

The experiments with [β-^{14}C]cystine indicate some conversion of cystine into serine. However, only 60 % of the total radioactivity in the serine was located in the β-carbon atom and the incorporation of ^{14}C into serine must therefore have taken place by an indirect pathway involving a redistribution of the isotope. The relative specific radioactivities of serine and alanine and the similar distribution of ^{14}C in the two amino acids are compatible with the formation of serine from alanine or pyruvate, with which alanine is known to be in rapid equilibrium by transamination. The conversion of cysteine to pyruvate has been demonstrated with extracts of rat liver (Smythe, 1942) and pyruvate is known to be converted into glycine by the rat, serine being a likely intermediate in this reaction (Anker, 1948). The distribution of ^{14}C in the alanine and serine, is satisfactorily explained by the participation of pyruvate in the reactions of the tricarboxylic acid cycle, which results in the isotope being distributed at random between the α- and β-carbon atoms of pyruvate and incorporation of ^{14}C into the carboxyl carbon atom by carbon dioxide fixation.

In agreement with the nutritional evidence described above, it is concluded from the present experiments that in the rat the utilization of serine (Stetten, 1942) and the sulphur of methionine (Du Vigneaud, Kilmer, Rachele & Cohn, 1944) for cysteine biosynthesis is not reversible, but it has not been established whether this is due to the lack of cystathionine synthesis from cysteine or to the absence of cleavage of cystathionine to serine and homocysteine. It is of interest in this connexion that cysteine is known to be a precursor of serine and methionine in *Neurospora* (Horowitz, 1947).

The extremely low radioactivity in choline and methionine after feeding [β-^{14}C]cystine shows that the β-carbon atom of cystine does not give rise to a one-carbon compound, such as formaldehyde or formate, which would be a precursor of methyl groups.

The major pathway of cysteine metabolism in the rat appears to involve the formation of pyruvate, which results in a high radioactivity in the alanine of the visceral protein and in the acetyl-carbon atoms of the excreted α-acetamido-γ-phenylbutyric acid. A direct conversion of cysteine to alanine rather than pyruvate is not ruled out by the present results, but it has recently been shown that pyruvate is formed enzymically from cysteine via cysteine-sulphinic and β-sulphinopyruvic acids (Kearney & Singer, 1952). The formation of pyruvate from cystine is also indicated by the extensive incorporation of ^{14}C into glutamic and aspartic acids, since these two amino acids are in equilibrium with α-ketoglutarate and oxaloacetate respectively, which are metabolically closely related to pyruvate by the reactions of the tricarboxylic acid cycle. Pyruvate is known to acetylate α-amino-γ-phenylbutyric acid (Anker, 1948, 1950) and its quantitative importance in cysteine metabolism may therefore be assessed by comparing the efficiency of cystine as a precursor of the acetyl group of α-amino-γ-phenylbutyric acid with that of compounds known to be converted to pyruvate *in vivo* or with that of sodium acetate which is a very effective precursor of acetyl groups (Anker, 1948, 1950). Such a comparison shows that DL-cystine is only slightly less effective as a source of acetyl groups than DL-valine, which is known to be extensively converted to 3-carbon acids, probably propionate and pyruvate (Peterson, Fones & White, 1952; Fones, Waalkes & White, 1951), or sodium acetate (cf. Table 3) or DL-serine (Arnstein, 1951*a*).

In the present experiments the labelled cystine and valine were fed as the DL-amino acids and it is possible that some of the above conclusions are not strictly applicable to the metabolism of the optically active enantiomorphs. However, it has been shown that in the rat the overall metabolism of D- and L-valine (Rose, Johnson & Haines, 1942) and the rate of oxidation of the sulphur of L- and DL-cystine (Lawrie, 1932) are similar. Furthermore, the rat is able to utilize D-valine for growth (White, Fones & Sober, 1952). D-Cystine is not utilized by the rat for growth (Du Vigneaud, Dorfmann & Loring, 1932), but this is probably due to the fact that the corresponding keto acid is destroyed faster than it is converted to L-cysteine (Stekol, 1948) and not to different metabolic pathways of the two enantiomorphs. It seems likely therefore that the intermediary metabolism of L-cystine and L-valine is at least qualitatively similar to that of the corresponding DL-amino acids.

SUMMARY

1. The metabolism of DL-cystine by the rat has been studied by feeding DL-[^{35}S]cystine and DL-[β-^{14}C]cystine.

2. The relatively poor conversion of the carbon-labelled cystine into serine and the almost complete absence of radioactivity in methionine after feeding sulphur-labelled cystine indicates that the formation of cystine from methionine and serine via cystathionine is not reversible to any significant extent.

3. There was no appreciable conversion of the β-carbon atom of cystine to the methyl groups of choline or methionine. A similar experiment with DL-[γ-^{14}C]valine showed that this amino acid also was not a precursor of methyl groups.

4. The major pathway of cysteine metabolism appears to be to pyruvate as shown by the good incorporation of ^{14}C from dietary DL-[β-^{14}C]cystine into alanine, aspartic acid and glutamic acid of the visceral protein and into the acetyl group of α-acetamido-γ-phenylbutyric acid excreted in the urine.

5. A synthesis of DL-[β-^{14}C]cystine from $^{14}CH_3OH$ is described.

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REFERENCES

Anker, H. S. (1948). *J. biol. Chem.* **176**, 1337.
Anker, H. S. (1950). *J. biol. Chem.* **187**, 167.
Anslow, W. P., Simmonds, S. & Du Vigneaud, V. (1946). *J. biol. Chem.* **166**, 35.
Armstrong, M. D. & Binkley, F. (1948). *J. biol. Chem.* **174**, 889.
Arnstein, H. R. V. (1951*a*). *Biochem. J.* **49**, 439.
Arnstein, H. R. V. (1951*b*). *Biochem. J.* **48**, 27.
Arnstein, H. R. V. & Neuberger, A. (1953*a*). *Biochem. J.* **55**, 271.
Arnstein, H. R. V & Neuberger, A. (1953*b*). *Biochem. J.* **55**, 259.
Campbell, P. N. & Work, T. S. (1952). *Biochem. J.* **50**, 449.
Chargaff, E. & Sprinson, D. B. (1943*a*). *J. biol. Chem.* **151**, 273.
Chargaff, E. & Sprinson, D. B. (1943*b*). *J. biol. Chem.* **148**, 249.
Crawhall, J. C. & Elliott, D. F. (1951). *J. chem. Soc.* p. 2071.
Dakin, H. D. (1913). *J. biol. Chem.* **14**, 321.
Du Vigneaud, V., Cohn, M., Chandler, J. P., Schenck, J. R. & Simmonds, S. (1941). *J. biol. Chem.* **140**, 625.
Du Vigneaud, V., Dorfmann, R. & Loring, H. S. (1932). *J. biol. Chem.* **98**, 577.
Du Vigneaud, V., Kilmer, G. W., Rachele, J. R. & Cohn, M. (1944). *J. biol. Chem.* **155**, 645.
Ehrensvärd, G., Reio, L., Saluste, E. & Stjernholm, R. (1951). *J. biol. Chem.* **189**, 93.
Fones, W. S., Waalkes, T. P. & White, J. (1951). *Arch. Biochem. Biophys.* **32**, 89.
Fromageot, C. & Clauser, H. (1949). *Biochim. biophys. Acta*, **3**, 422.
Gale, E. F. & Stephenson, M. (1938). *Biochem. J.* **32**, 392.
Horowitz, N. H. (1947). *J. biol. Chem.* **171**, 255.
Kearney, E. B. & Singer, T. P. (1952). *Biochim. biophys. Acta*, **8**, 698.
Knox, W. E. & Mehler, A. H. (1950). *J. biol. Chem.* **187**, 419.
Lawrie, N. R. (1932). *Biochem. J.* **26**, 435.
Lynen, F., Reichert, E. & Rueff, L. (1951). *Liebigs Ann.* **574**, 1.
Martin, E. L. & Fieser, L. F. (1943). *Org. Synth.*, Coll. **2**, p. 570. Ed. by A. H. Blatt. New York: John Wiley and Sons, Inc.; London: Chapman and Hall Ltd.
Neuberg, C. & Kerb, J. (1912). *Biochem. Z.* **40**, 498.
Peterson, E. A., Fones, W. S. & White, J. (1952). *Arch. Biochem. Biophys.* **36**, 323.
Pirie, N. W. (1932). *Biochem. J.* **26**, 2041.
Popják, G. (1950). *Biochem. J.* **46**, 560.
Reid, J. C. & Landefeld, M. O. (1951). *Arch. Biochem. Biophys.* **34**, 219.
Rose, W. C., Johnson, J. E. & Haines, W. J. (1942). *J. biol. Chem.* **145**, 679.
Sakami, W. (1950). *J. biol. Chem.* **187**, 369.
Simpson, M. V. & Tarver, H. (1950). *Arch. Biochem.* **25**, 384.
Smythe, C. V. (1942). *J. biol. Chem.* **142**, 387.
Soucy, R. & Bouthillier, L. P. (1951). *Rev. canad. Biol.* **10**, 290.
Stein, W. H. & Moore, S. (1950). *Cold Spr. Harb. Symp. quant. Biol.* **14**, 179.
Stekol, J. A. (1948). *J. biol. Chem.* **176**, 33.
Stekol, J. A. & Weiss, K. (1950). *J. biol. Chem.* **186**, 343.
Stetten, D. (1942). *J. biol. Chem.* **144**, 501.
Toporek, M., Miller, L. L. & Bale, W. F. (1952). *J. biol. Chem.* **198**, 839.
White, J., Fones, W. S. & Sober, H. A. (1952). *J. biol. Chem.* **199**, 505.

The Carbohydrates of *Agave vera cruz* Mill.

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In a preliminary communication from this Laboratory, Srinivasan, Bhalerao & Subramanian (1952) reported the presence of polyfructosans in the stem of *Agave vera cruz* (hereinafter referred to as the agave; for description of this species, see Firminger, 1905; Duthie, 1915; Bailey, 1919). This material has since been shown by chromatographic methods to contain a number of other water-soluble carbohydrates (Bhatia, Satyanarayana & Srinivasan, 1953). The carbohydrate make-up in the stem of the agave appears, in general, to be similar to that reported by Dedonder (1950) and by Bacon & Edelman (1951) in Jerusalem artichoke. However, neither of the two polyfructosans isolated from the agave seems to be identical with inulin.

EXPERIMENTAL

Material. The plants used were all from wild growth in the fields adjoining Mysore South. The leaves and the root-end were discarded and the stem, after scraping off the fibrous skin, cut into small pieces and shredded in a brass grater. The grated material was squeezed through muslin by hand. The juice was heated on a water bath till it coagulated completely and then allowed to settle. The supernatant solution was centrifuged and filtered. The filtrate, an opaque, pale-yellow fluid, had a pH varying from 5 to 6. It contained malic acid (detected by chromatography). The following tests were positive: test for phenolic substances (with diazotized aniline), Molisch's test, Fehling's test (weak reduction) and Seliwanoff's test (strongly positive). Starch was absent.

Methods for the determination of sugars. Hydrolysis was carried out with N-HCl for 9 min. at 67°. Total reducing sugar (T.R.S.) was determined by the method of Lane & Eynon (1923) and expressed as 'fructose'. Aldose was determined by the hypoiodite method of Klein & Acree (1930) and the results expressed as 'glucose'. Optical activity was measured at 25° in a 1 dm. tube and with a sodium lamp as the light source. Soluble solids in the juice (referred to as solids) were determined with the Abbé's refractometer (sugar scale); the juice was diluted to 10% solids before determining optical rotation.

Chromatographic methods. For reference spots, 1% solutions of known sugars were used. The juice was applied directly, i.e. after heat coagulation and filtration. The solvent was *n*-butanol:acetic acid:water (4:1:5, v/v) mixture (Partridge, 1948). The period of development was 4–10 days for the ascending chromatogram and about 16 hr. for the descending one. The benzidine trichloroacetic acid reagent, as recommended by Bacon & Edelman (1951), was used for spraying. Aldoses were located with the aid of aniline hydrogen phthalate (Partridge, 1949) and ketoses with the phloroglucinol reagent (Horrocks & Manning, 1949).

RESULTS

Variation in carbohydrate content of the plant

A survey of plant juices for their solids content and optical rotation was made with a view to choosing plants with maximum solids as starting material for the isolation of polyfructosans. Typical results of such a survey of about fifty plants selected at random over a period of about 10 months are presented in Table 1. Although there is no strict correlation between solids and rotation, plants having the maximum solids seem to possess also maximum laevorotation.

Table 1. *Variation in optical rotation and in solids from plant to plant of expressed juice from the stem*

Date of collection	α_D (°)	Solids range (%)
21–31. iii. 52	+1·85 to −3·5	16·8 to 31·0
8–14. iv. 52	+1·67 to −5·25	12·0 to 31·5
7–20. v. 52	−0·8 to −2·8	13·3 to 23·0
2–25. vi. 52	−0·45 to −5·95	7·5 to 21·7
14. vii. 52	−6·55, −6·85	30·0, 31·5
12–24. viii. 52	−6·1 to −10·0	29·5 to 34·0
12. ix. 52	+0·65, −4·5	8·3, 15·1
13. xii. 52	−9·22	31·5
i. 53	−9·1	28·7

Distribution of carbohydrates in the stem

This study was undertaken in order to select portions with most solids. Transverse sections were taken at different levels of the stem. Juice expressed from these sections was analysed and the results are given in Table 2. The optical rotations calculated on the basis of glucose and fructose of the hydrolysed juices were found to be in close agreement with the observed rotations (Table 2*A*, plant no. XII). The assumption that the sugars obtained on hydrolysis are glucose and fructose, needs, however, to be confirmed by further evidence based on specific estimations like that of glucose by glucose oxidase.

Carbohydrate gradient across the stem. In a particular cross-section, two portions were taken at widely separated distances thus: portion (1), from periphery down to 1·5 in. (3·8 cm.) all round;

Table 2. *Carbohydrate gradient along the stem: results obtained on three different plants*

(Experimental material: expressed juice from the different sections; α_D = optical rotation; T.R.S. = total reducing sugar; I–IV denote descending serial sections of the stem.)

	Initial		After hydrolysis					
					Fructose (T.R.S.–G/1·06)		α_D	
	α_D (°)	Solids (%)	T.R.S. (%)	Glucose (G) (%)	(%)	Fructose:glucose	Observed (°)	Calculated (°)*
(A) Plant no. XII/28. iv. 1952								
I	−1·85	15·1	14·0	4·35	9·9	69·5:30·5	−6·6	−6·66
II	−0·20	15·0	14·64	5·25	9·69	63·4:36·6	−5·8	−6·0
III	+0·49	14·2	12·2	4·91	7·57	60·7:39·3	−4·06	−4·26
IV	+1·5	12·5	10·4	5·05	5·64	52·8:47·2	−2·46	−2·44
(B) Plant no. XLI/8. xi. 1952								
I	−5·03	27·2	27·3	4·66	22·9	83·1:16·9	—	—
II	−5·03	27·2	28·6	4·74	24·13	83·6:16·4	—	—
III	−2·49	25·0	25·5	5·8	20·03	77·5:22·5	—	—
IV	−1·95	23·4	23·7	6·2	17·85	74·2:25·8	—	—
(C) Plant no. LV/27. i. 1953								
I	−9·64	−29·3	28·4	4·79	23·88	83·3:17·7	—	—
II	−8·74	−28·5	28·7	5·0	23·98	82·8:17·2	—	—
III	−8·7	27·75	26·4	4·05	22·58	84·8:16·2	—	—
IV	−9·36	29·1	28·4	4·55	24·11	84·1:15·9	—	—

* Based on $[\alpha]_D^{20°}$ glucose + 52·76° and for fructose −90·46°.

portion (2), central section 1·5 in. (3·8 cm.) in radius. The optical rotation and initial solids of the expressed juice from the two sections were as follows: (1) periphery: +1·67°, 12·0 %; (2) centre: −2·0°, 24·0 %.

Studies by chromatographic methods

The juice from the stem of the plant was analysed by ascending chromatography with the use of the following sugars as reference compounds: rhamnose, xylose, arabinose, fructose, mannose, sorbose, glucose, galactose, sucrose, maltose and raffinose. The spots were developed for 10 days by ascending flow of the solvent, dried and sprayed. The following sugars were detected in the juice: fructose, glucose, sucrose, four carbohydrates yet unidentified (possibly oligosaccharides) and polyfructosan (R_F, zero). Of the four unidentified carbohydrates (one more than previously reported by Bhatia *et al.* 1953), one had an R_F value higher than raffinose, while the other three had lower R_F values. Hydrolysis of the carbohydrates present in the juice yielded only two sugars: glucose and fructose. This has been shown in the following experiment. The juice was applied at two spots on the opposite bottom corners of the paper (40 × 40 cm.) and developed (ascending technique) for 4 days. After locating the chromatogram on a guide strip, the corresponding area over the other spot was hydrolysed with trichloroacetic acid by spraying with 10 % acid and heating at 110° for 10 min., and developed at right angles by the descending flow of the solvent for 16 hr., dried and sprayed with benzidine trichloroacetic acid reagent. It was then found that all the component spots of the juice had given rise, as a result of hydrolysis, to glucose and fructose.

Isolation of polyfructosans

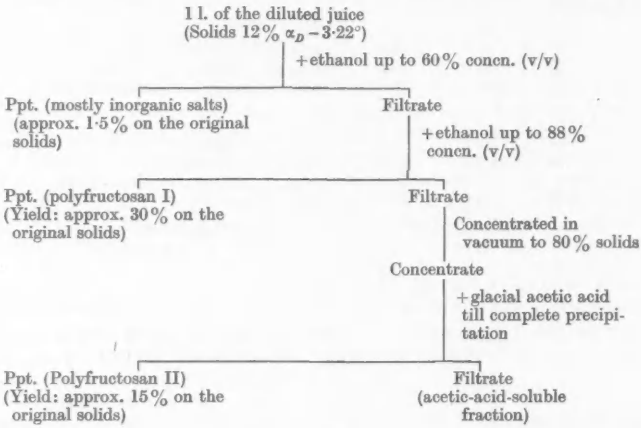
It was previously reported (Srinivasan *et al.* 1952) that hot extraction of the dried agave powder with methanol results in two fractions, one soluble and the other insoluble in this solvent, and that the two fractions have different optical rotations. In subsequent experiments to purify these two fractions, the aqueous solutions were first treated with ethanol up to 60 % concentration. Thereby a precipitate, with little organic matter in it, was obtained. On filtering off this precipitate and adding ethanol to the filtrate to 88 % concentration, a further precipitate was obtained. This was freed from ethanol and dried; $[\alpha]_D^{25°}$ of the material thus obtained from the methanol-insoluble fraction: initial, −38·0°; after hydrolysis, −82·0°; from the methanol-soluble fraction: initial, −37·2°; after hydrolysis, −83·5°.

Next, the isolation of polyfructosans directly from the expressed juice, without the use of methanol, was undertaken. The steps were as shown in scheme on p. 288.

Polyfructosan I. This is a snow-white powder, light and hygroscopic; completely water-soluble. After a few reprecipitations from 90 % ethanol, acetylation (Haworth, Hirst & Lyne, 1937) and deacetylation (Zemplen, 1926), it gave $[\alpha]_D^{25°}$ initial,

$-40{\cdot}0°$; after hydrolysis, $-88{\cdot}5°$. The deacetylated material was homogeneous chromatographically and gave rise only to fructose on hydrolysis.

Polyfructosan II. This is a snow-white powder and hygroscopic: it reduces Fehling's solution, but does not contain free reducing sugars. It does not move on the chromatogram and gives rise to both fructose and glucose after hydrolysis. Glucose released after hydrolysis amounts to 15·0%; $[\alpha]_D^{25°}$ initial, $-20°$; after hydrolysis, $-69°$. These characteristics remained unchanged after acetylation and deacetylation. Tentatively, this material also is referred to as a polyfructosan.


1 l. of the diluted juice
(Solids 12% α_D −3·22°)
+ethanol up to 60% concn. (v/v)
Ppt. (mostly inorganic salts)
(approx. 1·5% on the original solids)
Filtrate
+ethanol up to 88% concn. (v/v)
Ppt. (polyfructosan I)
(Yield: approx. 30% on the original solids)
Filtrate
Concentrated in vacuum to 80% solids
Concentrate
+glacial acetic acid till complete precipitation
Ppt. (Polyfructosan II)
(Yield: approx. 15% on the original solids)
Filtrate
(acetic-acid-soluble fraction)

DISCUSSION

Of the water-soluble carbohydrates in the stem of the agave, polyfructosans are the major constituents. The minor fractions contain sucrose and other carbohydrates composed of both glucose and fructose residues, besides free fructose and glucose. Though the carbohydrate content varies from plant to plant, the qualitative make-up of the carbohydrates appears to be the same; chromatograms obtained with different stem juices or with water extract of the dried material are similar (cf. Bhatia *et al.* 1953). The variations in the carbohydrate content (Table 1), have been recorded throughout a 10-month period. Also, when different plants were collected and examined on the same day, the stem juice of some showed a positive rotation (this only rarely), while in the majority the rotation was negative. It is, therefore, difficult to correlate these variations with a seasonal factor, contrary to what has been shown to be the case with Jerusalem artichoke (Thaysen, Bakes & Green, 1929; Bacon & Edelman, 1951; Bacon & Loxley, 1952). That this variation in the agave is possibly related to the age and maturity of the plant is indicated by results presented in Table 2. These results suggest that the content of the laevorotatory (fructose-containing) carbohydrates increases with maturity. This conclusion, however, needs to be confirmed, by a study of plants reared under known conditions. Plants used in our experiments were all collected from their native surroundings and were of unknown age, but far from their full maturity, since not one of them had 'poled'. Although the results reported here were all obtained with the stem of *Agave vera cruz* as the experimental material, possibly other species of the Agave family might give similar results. A trial chromatogram developed with the juice from the stem of *Fourcroya gigantea* appeared similar to one obtained with *Agave vera cruz*.

SUMMARY

1. Among the water-soluble carbohydrates in the stem of *Agave vera cruz* the following have been detected by chromatographic methods: fructose, glucose, sucrose, four other substances, possibly oligosaccharides and two polyfructosans.

2. The optical activity and the content of total soluble solids obtained from various plants vary a great deal. While generally the juice is laevorotatory, instances of dextrorotatory juices have been noticed.

3. In some plants, there is a gradient in the distribution of carbohydrates along the length of the stem, as also radially in a cross-section.

4. Preliminary details regarding the isolation and characteristics of two polyfructosans are described.

We are greatly indebted to Dr V. Subrahmanyan, Director of this Institute, for his interest and helpful suggestions. Our thanks are due to Mr M. N. Satyanarayana for much technical assistance and to the Economic Botanists of the States of Madras and Bombay and to Mr A. Sheriff for identifying the plant.

REFERENCES

Bacon, J. S. D. & Edelman, J. (1951). *Biochem. J.* **48**, 114.
Bacon, J. S. D. & Loxley, R. (1952). *Biochem. J.* **51**, 208.
Bailey, L. H. (1919). *The Standard Cyclopedia of Horticulture*, 3rd ed., p. 233. London: Macmillan.
Bhatia, I. S., Satyanarayana, M. N. & Srinivasan, M. (1953). *Curr. Sci.* **22**, 16.
Dedonder, R. (1950). *C. R. acad. Sci. Paris*, **230**, 997.
Duthie, J. F. (1915). *Flora of the Upper Gangetic Plain*, vol. III, pt. II, p. 240. Calcutta: Government Press.
Firminger, W. K. (1905). *A Manual of Gardening for India*, 6th ed., p. 24. Calcutta: Thacker Spinck.
Haworth, W. N., Hirst, E. L. & Lyne, R. R. (1937). *Biochem. J.* **31**, 786.
Horrocks, R. H. & Manning, G. B. (1949). *Lancet*, **1**, 1042.
Klein, G. M. & Acree, S. F. (1930). *Bur. Stand. J. Res., Wash.*, **5**, 1063.
Lane, J. H. & Eynon, L. (1923). *J. Soc. chem. Ind., Lond.*, **42**, 32T.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Partridge, S. M. (1949). *Nature, Lond.*, **16**, 443.
Srinivasan, M., Bhalerao, V. R. & Subramanian, N. (1952). *Curr. Sci.* **21**, 159.
Thaysen, A. C., Bakes, W. E. & Green, B. M. (1929). *Biochem. J.* **23**, 444.
Zemplen, G. (1926). *Ber. dtsch. chem. Ges.* **59** B, 1254.

The Determination of Lactic Acid in Microgram Quantities

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The conversion of lactic acid to acetaldehyde by hot concentrated sulphuric acid was first reported by Denigès (1909) and its use as a basis for the determination of lactic acid was described by Mendel & Goldscheider (1925). Eegriwe (1933) described a reaction between *p*-hydroxydiphenyl and acetaldehyde to yield a violet colour and this colour reaction was first adopted as a sensitive means of estimating lactic acid by Miller & Muntz (1938).

The method most widely employed for determining lactic acid on a microscale is that of Barker & Summerson (1941). In the course of work in this laboratory requiring the estimation of very small amounts of lactic acid, usually in the presence of pyruvate, reproducible results could not be achieved with this technique; further, the final optical densities were not directly proportional to the amounts of lactic acid present.

This paper contains the results of our investigations whereby several modifications of Barker & Summerson's procedure were made. With these changes, 1–8 μg. of lactic acid could be determined with an accuracy of $\pm 2\%$. When interfering pyruvic acid is present, the procedure involves a dilution so that the method in this case is applicable to 2–10 μg. lactic acid.

METHODS

Reagents

p-Hydroxydiphenyl (1·5 g.) is dissolved in 10 ml. of 5% (w/v) NaOH and diluted to 100 ml. with water.

Standard lactic acid solution: 0·2133 g. of pure, dry lithium lactate (Hillig, 1937), was dissolved in about 100 ml. of water, 1 ml. of concentrated H_2SO_4 (A.R.) added and the solution made up to 1 l. with water.

Recommended method

The protein-free solution (2 ml.) containing 10–80 μg. of lactic acid, is pipetted into a 150 × 25 mm. test tube which contains 1 ml. of 20% (w/v) $CuSO_4$, $5H_2O$ and the final volume is made up to 10 ml. with water. Approximately 1 g. of solid $Ca(OH)_2$ is added and, after thoroughly mixing and allowing to stand for 30 min. or more, the solution is centrifuged.

If the original 2 ml. of protein-free solution contains 100–600 μg. of pyruvic acid, 6 ml. of the supernatant solution from the first treatment with copper-lime is pipetted into a second tube containing 0·6 ml. of 20% (w/v) $CuSO_4$, $5H_2O$ and 0·6 g. of solid $Ca(OH)_2$ added. This procedure is then repeated a third time with 3 ml. of supernatant solution, 0·3 ml. of 20% $CuSO_4$, $5H_2O$ and 0·3 g. of $Ca(OH)_2$, whereby the amount of pyruvic acid present is reduced to a minimum and the colour interference due to it becomes a small constant value which may be deducted from the final optical density.

1 ml. of the supernatant solution from the third copper-lime treatment is transferred to another 150 × 25 mm. test tube, held in the arm of a mechanical shaker with its lower end immersed in an ice-water mixture. $CuSO_4$, $5H_2O$ (0·05 ml. of 12% (w/v) solution) is added followed by 6 ml. of conc. H_2SO_4 (A.R.) dropwise from a burette with vigorous shaking, the tap of the burette being lubricated with the concentrated acid. After complete addition, the contents are poured and allowed to drain into a small ground-glass stoppered Pyrex tube which is then heated for 30 min. in a water bath maintained at 60 ± 1°. The tube is allowed to

cool to 10–15°, the stopper removed, 0·1 ml. of the *p*-hydroxydiphenyl reagent added and the precipitated *p*-hydroxydiphenyl thoroughly dispersed in the H_2SO_4. Incubation of the tube for 20 min. at 28–30° to ensure maximum colour development is then followed by 90 sec. in a boiling-water bath to destroy excess *p*-hydroxydiphenyl. After this treatment the tube is immediately cooled in ice-water and the optical density of the resulting violet-coloured solution is determined in circular tubes of 1 cm. diameter with the Unicam diffraction-grating spectrophotometer at a wavelength peak of 560 mμ. against a reagent blank prepared by taking distilled water through the whole of the above procedure.

The calibration curve obtained by this method ($E = \mu g.$ lactic acid × 0·059) for 0–8 μg. of lactic acid is a straight line.

DEVELOPMENT OF METHOD

The effect of varying the experimental conditions at different stages of the method is described in Table 1. The absorption curve of the acetaldehyde-*p*-hydroxydiphenyl complex shows a sharp maximum at 560 mμ. as illustrated by the following optical densities obtained from 6 μg. of lactic acid in the conditions described on p. 289 at wavelengths between 520 and 600 mμ.: 520, 0·210; 540, 0·295; 550, 0·330; 560, 0·345; 570, 0·330; 580, 0·305; 600, 0·185.

The calibration curve, which is strictly reproducible, indicates that the coloured complex obeys the Lambert-Beer Law in the concentration range of 1–8 μg. of lactic acid. Above 8 μg. the optical density does not increase in direct proportionality with the amount of lactic acid but falls off slightly. If coloured solutions, obtained from larger quantities of lactic acid (up to 10 μg.), are diluted with H_2SO_4: water (6:1, v/v) the optical densities of the diluted solutions are directly proportional to the amounts of lactic acid originally present.

Specificity of the method

The work for which the method has been used required the determination of lactic acid in the presence of acetoin, butane-2:3-diol, diacetyl and pyruvate. None of the first three of these compounds gives any colour when the method is applied to solutions containing 60 μg. of it and quantitative recoveries of lactic acid are always obtained in the presence of such amounts. However, pyruvic acid, which is commonly present with lactic acid in media from enzymic studies, seriously interferes with the reproducibility of the method unless steps are taken to remove it. This is illustrated by the results collected in Table 2 for pyruvic acid samples without copper-lime treatment.

One copper-lime treatment does not yield reproducible results, although it clearly removes a considerable amount of pyruvic acid. The repetition of the copper-lime procedure seemed a likely way of completely eliminating the interference due to pyruvate and the effect of three successive treatments is also illustrated by the results in Table 2. The third treatment reduces the optical density of the interfering pyruvic acid to a small constant value. Using this technique, recoveries of lactic acid ranging from 98 to 102% may be obtained in mixtures containing 2–10 μg. of lactic acid and 10–60 μg. of pyruvic acid/ml. of final solution;

Table 1. *Effects of varying experimental conditions*

('Colour' means optical density at 560 mμ.)

Stage	Condition varied	Recommended procedure	Effect of other conditions
Oxidation	Heating at 100° in open tube	—	The longer the heating, the less colour obtained
Oxidation	Stoppered and unstoppered tubes	Stoppered tubes	Unstoppered tubes give about 15% less colour
Oxidation	Time of heating at 60°	30 min.	24–65 min. gives maximum colour; <24 min., less colour
Oxidation	Concn. of Cu^{2+} added	0·05 ml. of 12% (w/v) $CuSO_4, 5H_2O$	12–32%, same colour; <12%, less colour
Incubation with *p*-hydroxydiphenyl reagent	Time at 28–30°	20 min.	20–50 min., same colour; <20 or >50 min., less colour
Destruction of excess *p*-hydroxydiphenyl	Time of heating at 100°	90 sec.	30–120 sec., identical colour

Table 2. *The effect of successive copper-lime treatments on the colour obtained with pyruvic acid samples*

((I) 9 ml. of standard solutions (containing 200, 300, 400, 500 and 600 μg. of pyruvic acid) + 1 ml. of 20% $CuSO_4, 5H_2O$ + 1 g. $Ca(OH)_2$. (II) 6 ml. of supernatant solution from (I) + 0·6 ml. 20% $CuSO_4, 5H_2O$ + 0·6 g. $Ca(OH)_2$. (III) 3 ml. of supernatant solution from (II) + 0·3 ml. 20% $CuSO_4, 5H_2O$ + 0·3 g. $Ca(OH)_2$. 1 ml. of supernatant solution taken for analysis in each case.)

Pyruvic acid concn. (μg./ml. in final solution)	Optical densities			
	Without treatment	(I) After one copper-lime treatment	(II) After two copper-lime treatments	(III) After three copper-lime treatments
20	0·200	0·070	0·020	0·017
30	0·275	0·105	0·030	0·020
40	0·38	0·110	0·050	0·023
50	0·43	0·125	0·050	0·022
60	0·52	0·130	0·055	0·020

Table 3. *Recoveries of lactic acid from mixtures of pyruvic and lactic acids*

Final solution: Lactic acid (μg./ml.)	Final solution: Pyruvic acid (μg./ml.)	Optical density of mixture (Obs.)	Optical density (Corr.*)	Recovery of lactic acid (%)
2	60	0·140	0·120	101
4	60	0·265	0·245	102
6	60	0·368	0·348	98
8	60	0·490	0·470	99
6	20	0·385	0·365	103
6	30	0·375	0·355	100
6	40	0·380	0·360	101
6	50	0·368	0·348	98
6	60	0·375	0·355	100

* Observed value *minus* 0·02 which is the average optical density produced by remaining pyruvic acid (20–60 μg./ml. final solution).

these recoveries are corrected for the small constant optical density due to interfering pyruvic acid after three copper-lime treatments. The results are shown in Table 3.

Similar recoveries are obtained when known quantities of lactic acid are added to tissue and enzyme extracts and deproteinization carried out with 10% (w/v) trichloroacetic acid.

DISCUSSION

The conditions under which the oxidation of lactic acid to acetaldehyde is carried out, in the first stage of the method of estimation, are of critical importance. When this oxidation is carried out in open tubes, the acetaldehyde recoverable as the final coloured complex varies greatly with the time of heating employed. These findings are contrary to those of Barker & Summerson (1941) who state that identical results are obtained with heating periods of 3–10 min. Mendel & Goldscheider (1925), whose method of lactic acid estimation used 1:2-dimethoxybenzene instead of *p*-hydroxydiphenyl to give a red compound, also claimed that heating in open tubes for 4–8 min. during the sulphuric acid oxidation stage produced no differences in the optical density of the resulting coloured complex. However, Miller & Muntz (1938) did recommend the use of stoppered tubes, but gave no reason for this.

Our results confirm those of Barker & Summerson regarding the influence of copper on the lactic acid oxidation but we have found it necessary to use a larger amount (0·05 ml. of 12% $CuSO_4$, $5H_2O$ instead of 0·05 ml. of a 4% solution) to achieve the full increase in sensitivity. This modification may be required owing to the greater recoveries of lactic acid as acetaldehyde using stoppered tubes.

The modification of method necessary to estimate lactic acid accurately in the presence of pyruvic acid was suggested by the work of Van Slyke (1917). Miller & Muntz (1938) have suggested heating the sulphuric acid oxidation mixture for 15 min. instead of 5 min. in order to remove any interfering pyruvate.

SUMMARY

1. Lactic acid has been determined in the range 0–8 μg./ml. with an accuracy of ± 2%.

2. The method, which can be employed over a 2–10 μg./ml. range in the presence of 10–60 μg./ml. of pyruvic acid, is based on that of Barker & Summerson (1941), but certain modifications are incorporated to achieve reproducibility.

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REFERENCES

Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
Denigès, G. (1909). *Bull. Soc. chim. Fr.* **5**, 647.
Eegriwe, E. (1933). *Z. anal. Chem.* **95**, 323.
Hillig, F. (1937). *J. Ass. off. agric. Chem., Wash.*, **20**, 130.
Mendel, B. & Goldscheider, I. (1925). *Biochem. Z.* **164**, 163.
Miller, B. F. & Muntz, J. A. (1938). *J. biol. Chem.* **126**, 413.
Van Slyke, D. D. (1917). *J. biol. Chem.* **32**, 455.

Studies in the Bile Acids

2. THE NON-KETONIC ACIDS OF HUMAN BILE

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A method of analysis has been described in which a mixture of bile acids from bile can be separated and estimated by a combination of chromatography and infrared spectrometry (Wootton, 1953). This method was applied to a specimen of pooled human bile kindly supplied by the late Dr Konrad Dobriner. To our surprise, we found that the most abundant bile acid in the specimen was chenodeoxycholic acid (3α:7α-dihydroxycholanic acid), although this acid is usually regarded as one of the minor constituents of mammalian bile.

This particular specimen had been collected at autopsy from patients who had suffered from advanced carcinomatosis, and it was considered possible that their clinical condition had influenced the constitution of the bile. It was therefore decided to analyse individual specimens from patients with and without carcinoma. It was also felt that such a study would help to define the natural variability of the proportions of the different bile acids in the human, a matter which is possibly of importance in the aetiology of gall-stones. Furthermore, the use of bile constituents as an 'evolutionary label' (Haslewood & Wootton, 1950) has created interest in the variability within a single species.

In the course of this work, chenodeoxycholic acid was produced from cholic acid (3α:7α:12α-trihydroxycholanic acid) on a preparative scale by a method which has been briefly reported (Anderson, Haslewood, Wiggins & Wootton, 1952). Several esters were made and characterized.

EXPERIMENTAL

Material. Bile specimens were obtained from nine patients at autopsy. Within a few hours of death, the contents of the gall-bladder were mixed with 2 vol. of ethanol. One specimen (case XII) was collected during life from a patient with bile fistula following cholecystectomy; in this case the bile was run directly into a bottle containing ethanol.

Preparation of esters. The bile-ethanol mixture was filtered from precipitated mucin and the filtrate evaporated to dryness on a steam bath and finally under vacuum. The residue, dissolved in water, was mixed with half its weight of charcoal and dilute NaOH solution added until a spot test gave a pink colour with phenolphthalein. After drying at 100°, the powdered solid was extracted with ethanol in a continuous extractor and the ethanol was removed under vacuum.

The ethanol-soluble bile salts (1 g.) were dissolved in 10 ml. 2·5 N-NaOH, heated to 120° overnight in a closed bomb and the reaction mixture diluted and acidified. The precipitated bile acids were collected, washed with water and dried. They were dissolved in methanol containing 1% (v/v) H_2SO_4 and allowed to stand for 2 days at room temperature, the solution was then diluted with water and extracted with ether. The ether extracts were washed with dilute ammonia solution and water, dried (Na_2SO_4) and evaporated, yielding an amber gum containing the mixed methyl esters of the bile acids.

Separation and identification. The esters were separated on columns of silica gel and the individual fractions were examined and identified by infrared spectrometry as described by Wootton (1953). The instrument used was a single-beam Grubb-Parsons S3A with a NaCl prism.

Fractions displaying similar spectra were combined, so that in general each specimen was divided into parts comprising the methyl esters of (*a*) lithocholic acid (3α-hydroxycholanic acid), (*b*) a mixture of chenodeoxycholic acid and deoxycholic acids (3α:7α- and 3α:12α-dihydroxycholanic acids), and (*c*) cholic acid (3α:7α:12α-trihydroxycholanic acid). The amounts of the two esters present in (*b*) were determined in the following way. A number of mixtures were made containing different proportions of the pure synthetic methyl esters of chenodeoxycholic acid and deoxycholic acid. The infrared spectrum of each mixture was obtained and a celluloid transparency prepared from it. The spectrum of the fraction from the column was then compared with the set of transparencies by three independent observers. An analysis of the results indicated that the percentage of each ester could be estimated with a standard deviation of 3·8%.

Reference compounds

General. Melting points were measured with a hot-stage microscope and are corrected. Optical rotations were determined on solutions in $CHCl_3$ at about 20° using a 0·5 dm. tube and concentrations of 0·8–1·0%. Microanalyses were done by Weiler and Strauss, Oxford. The light petroleum had b.p. 40–60. 20% Chromic acid was made as described by Haslewood & Wootton (1950). The infrared spectra were taken in CS_2 solution; concn. about 2 mg./ml., solution thickness 3 mm. These are shown in Fig. 1.

Chenodeoxycholic acid. This preparation was accomplished by the reduction of 3:7-diacetoxy-12-ketocholanic acid by the method of Huang-Minlon (1946). The starting material (4·3 g., m.p. 226–228°) was dissolved in a mixture

of 40% KOH (12·5 ml.) ethylene glycol (150 ml.) and 90% hydrazine hydrate solution (5 ml.). After refluxing for 1·5 hr., the condenser was removed and heating continued until the b.p. rose to 195°. Refluxing was then continued for 4 hr. The reaction mixture was diluted and saturated with NaCl and the crude chenodeoxycholic acid was collected as an amorphous white powder (3·0 g.).

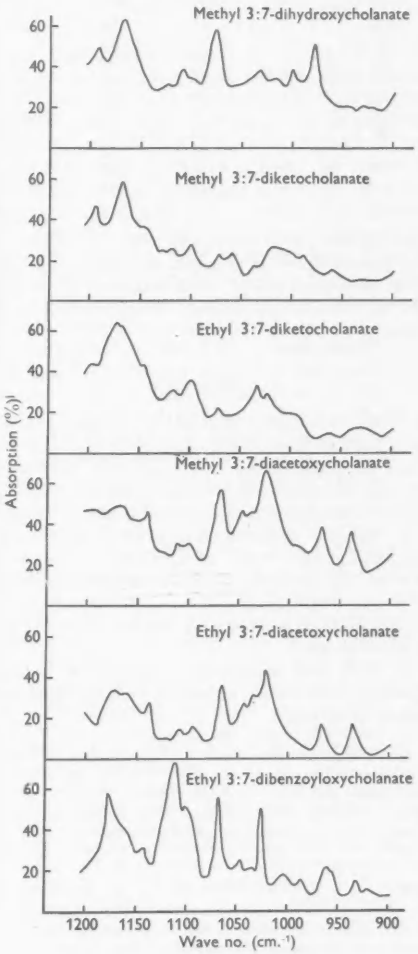

Fig. 1. Infrared-absorption spectra of derivatives of 3:7-diketo- and 3:7-dihydroxy-cholanic acid.

Methyl chenodeoxycholate. The acid (0·5 g.) was dissolved in 50 ml. methanol containing 1% (v/v) H_2SO_4. After 48 hr. at room temperature, the solution was diluted and extracted with ether. After washing, drying and evaporating the ether, a colourless gum remained. After solution in ether:light petroleum *methyl chenodeoxycholate* (0·45 g.) crystallized slowly as sheaves of colourless needles, m.p. 85–87° $[\alpha]_D$ +12°. (Found: C, 72·7; H, 10·2. $C_{25}H_{42}O_4$ requires C, 73·8; H, 10·4%.) Several preparations which were analysed had low C content. On one occasion, sublimation in high vacuum yielded a small amount of crystalline material, m.p. 113–114°, with an unchanged infrared spectrum. It is concluded that the normal form contains solvent of crystallization.

A number of attempts were made to prepare the ethyl ester by a similar method. No crystalline material could be recovered.

Methyl diacetylchenodeoxycholate (*methyl 3α:7α-diacetoxycholanate*). Methyl chenodeoxycholate (0·05 g.) was dissolved in a mixture of 4 ml. acetic acid, 1·5 ml. acetic anhydride and 0·05 ml. 60% (w/v) $HClO_4$. After standing for 0·5 hr. at room temperature, the diluted mixture was extracted with ether. The gummy residue remaining after evaporation of the washed and dried ether was dissolved in a mixture of ether:light petroleum (3:7, v/v) and passed through a column of silica gel. The recovered material crystallized from light petroleum as white prisms (0·03 g.), m.p. 127–128°, $[\alpha]_D$ +14°. (Found: C, 71·1; H, 9·1. Calc. for $C_{29}H_{46}O_6$: C, 71·0: H, 9·4%.)

Ethyl diacetylchenodeoxycholate (*ethyl 3α:7α-diacetoxycholanate*). This was made in a similar way, starting with the gummy ethyl ester. It crystallized in a similar form, m.p. 106–108°, $[\alpha]_D$ +12°. (Found: C, 71·5; H, 9·2. Calc. for $C_{30}H_{48}O_6$: C, 71·4, H, 9·5%.) The m.p. was unchanged after admixture with the natural ester, m.p. 106–108°, isolated from natural sources and kindly supplied by Prof. G. A. D. Haslewood.

Methyl 3:7-diketocholanate. Methyl chenodeoxycholate (0·03 g. in 2 ml. acetic acid) was oxidized by the dropwise addition of 20% chromic acid until a permanent brown colour remained. After 20 min., the mixture was diluted with water. Long needles slowly separated, m.p. 160–162°, raised by recrystallization from aqueous ethanol to 162–164° (0·02 g.) $[\alpha]_D$ −36°. (Found: C, 74·2; H, 9·6. Calc. for $C_{25}H_{38}O_4$: C, 74·6; H, 9·5%.)

Ethyl 3:7-diketocholanate. This was prepared in the same way from the ethyl ester. The *ethyl 3:7-diketocholanate* crystallized as long needles, m.p. 126–129°. $[\alpha]_D$ −38°. (Found: C, 74·8; H, 9·8. $C_{26}H_{40}O_4$ requires C, 75·0; H, 9·7%.)

Ethyl dibenzoylchenodeoxycholate (*ethyl 3α:7α-dibenzoyloxycholanate*). The ethyl ester (0·1 g.) in pyridine (1 ml.) and benzoyl chloride (0·2 ml.) was allowed to remain overnight at room temperature. The product was washed by decantation with dilute H_2SO_4 and dissolved in ether. The residue after evaporating the washed ether was taken up in benzene:light petroleum (1:1, v/v) and passed through a column of Al_2O_3. The gummy ester was left in contact with ether:light petroleum and crystallized after several months. Recrystallized from ether:light petroleum, the *ethyl dibenzoylchenodeoxycholate* formed long needles (0·075 g.) m.p. 99–102°, $[\alpha]_D$ −31°. (Found: C, 75·4; H, 8·2. $C_{40}H_{52}O_6$ requires C, 76·5; H, 8·3%.)

RESULTS

The proportions of each of the four non-ketonic acids present in human bile are given in Table 1, which also shows the ages of the patients and the pathological conditions found at autopsy (except on

Table 1. *Proportions of the different bile acids in human bile specimens*

Case no.	Pathological condition	Age (years)	Acids (as % of total) Litho-cholic acid	Deoxy-cholic acid	Chenodeoxy-cholic acid	Cholic acid
I	Coronary thrombosis	83	—	28	44	28
III	Gastric carcinoma and liver metastases	60	—	6	49	45
VI	Intestinal obstruction	46	—	18	40	42
VII	Tuberculous meningitis	65	5	23	34	38
VIII	Subarachnoid haemorrhage	61	—	28	37	35
IX	Sarcoma of the ileum and liver metastases	54	—	3	37	60
X	Carcinoma of lung and liver metastases	65	—	28	42	30
XI	Pulmonary tuberculosis	26	—	17	51	32
XII	Bile fistula after cholecystectomy	46	—	8	32	60
XIII	Gastroenteritis	3	—	12	47	41

case XII which was a post-operative specimen). The amounts found were rather variable, but on inspection there is some semblance of a pattern. Lithocholic acid was only detected in one specimen. Chenodeoxycholic acid was abundant and constituted 30–50 % of the total. The remainder seems to be distributed between deoxycholic acid and cholic acid, and, when the former was scarce, the latter was correspondingly abundant.

DISCUSSION

These results amply confirm our previous impression that chenodeoxycholic acid is a major constituent of human bile. The large quantities present have presumably been overlooked because this substance is exceedingly difficult to isolate by classical techniques, and, moreover, many reported analyses depend on colour reactions of very doubtful specificity. It is, however, noteworthy that Murarami (1952) has found very similar amounts by a colorimetric method which distinguishes only dihydroxy and trihydroxy acids.

There appears to be no reason to suppose that carcinomatosis affects the proportions of the different acids in any way, nor is there any obvious correlation with age. It remains to be determined whether this pattern is in any way characteristic of the human species, or whether other mammalian biles, now thought to contain mainly cholic acid, will be found to be similar. Further work on this point is in progress.

SUMMARY

1. A convenient method of preparing chenodeoxycholic acid (3α:7α-dihydroxycholanic acid) from 3α:7α-diacetoxy-12-ketocholanic acid is described. Several esters of chenodeoxycholic acid are characterized and their infrared spectra given.

2. Analysis of human autopsy bile specimens by chromatography and infrared spectrometry has shown that chenodeoxycholic acid is an abundant constituent, while lithocholic acid is scarce. There is no obvious connexion with age or the presence of carcinomatosis.

3. It is still uncertain whether this finding is specific to the human species or is part of a common mammalian plan.

We wish to thank Prof. E. J. King for his support and encouragement, Dr C. V. Harrison for providing bile specimens, and Prof. G. A. D. Haslewood for supplying a sample of fistula bile. Miss Audrey Robinson and Miss Pauline Lewis provided technical assistance.

REFERENCES

Anderson, I. G., Haslewood, G. A. D., Wiggins, H. S. & Wootton, I. D. P. (1952). *Nature, Lond.*, **169**, 621.
Haslewood, G. A. D. & Wootton, V. (1950). *Biochem. J.* **47**, 584.
Huang-Minlon (1946). *J. Amer. chem. Soc.* **68**, 2487.
Murarami, E. (1952). *J. Biochem., Tokyo*, **39**, 17.
Wootton, I. D. P. (1953). *Biochem. J.* **53**, 85.

7-Dehydrosterol in the Sexual Organs of the Rat

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In a study of the distribution of vitamin A in the rat we examined the male reproductive system under ultraviolet irradiation screened with Wood's glass (Moore & Ward, 1951). Faint-yellow fluorescence suggested the presence of the vitamin in the caput epididymis and preputial glands of normally nourished animals. Attempts to confirm this conclusion by spectrophotometric methods, however, were impeded by the presence of well-defined absorption bands at 293, 282, 272 and 262 mμ., characteristic of 7-dehydrocholesterol or ergosterol. The intensity of the absorption suggested that one of these substances was present in unusually high concentration. It seemed of interest, therefore, to investigate whether the amount of the sterol could be influenced by dietary changes, including deficiency of vitamin A, and whether it could also be detected in the sexual organs of other species.

A preliminary account of our main findings has already been given (Moore & Ward, 1952). The purpose of the present communication is to give details of our spectroscopic data, and to report tests on an irradiated extract of rats' preputial glands for antirachitic activity. Since we have not isolated the substance responsible for the spectrophotometric absorption in pure form, and in amounts sufficient for its identification, we refer to it as 7-dehydrosterol. We consider that it is almost certainly 7-dehydrocholesterol, as implied by previous workers who have observed the same characteristic absorption spectrum in extracts made from tissues of higher animals (Glover, Glover & Morton, 1952).

EXPERIMENTAL

Methods. The organs under investigation were mostly taken from piebald rats, and were carefully dissected free from adipose tissue. In our earlier experiments they were first ground with quartz powder and anhydrous Na_2SO_4, and extracted in the cold with several portions of ether. The combined extracts were then evaporated to dryness under reduced pressure, and the fat was redissolved in a measured volume of ether for a preliminary examination with a Unicam S.P. 500 spectrophotometer. After the solvent had again been removed the fat was saponified on a boiling-water bath with 0·44 ml. of a saturated aqueous solution of KOH and 2 ml. of ethanol/g. fat. The unsaponifiable residue, dissolved in light petroleum (b.p. 40–60°) was fractionated by chromatographic adsorption on a column (1 cm. diameter, 5 cm. length) of Al_2O_3 (Brockmann, Grade 2) followed by elution with 100 ml. portions of light petroleum containing increasing quantities of ether. In later experiments the preliminary extraction with ether was omitted, and the tissues were digested directly in hot 25% ethanolic KOH.

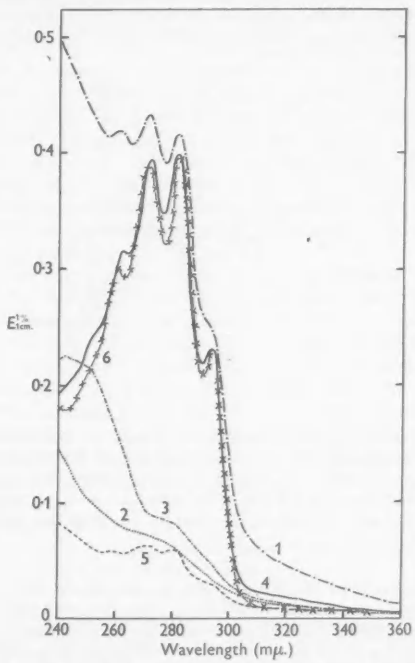

Fig. 1. Ultraviolet absorption spectra in ether of fractions obtained from the preputial glands of piebald rats by chromatography on alumina. 1, crude unsaponifiable matter ($\frac{1}{4}$ scale). 2–5, eluates obtained with light petroleum containing ether; 2, 5% ether; 3, 10% ether; 4, 20% ether; 5, 30% ether. 6, Pure 7-dehydrocholesterol reduced to same scale as 4.

With each procedure the extracts were usually examined spectrophotometrically at each stage. Extinction coefficients $(E)_{1\,cm.}$ have been calculated on the basis of an extract from 1 g. of the original tissue being dissolved in 100 ml. of solvent. It will be understood that E falls as the 7-dehydrosterol is lost during purification or divided between different fractions.

Fig. 1 shows how chromatography increased the clarity of the absorption spectrum of 7-dehydrosterol by the removal

of substances having non-selective absorption. The extract in this experiment was made from the preputial glands of male rats by direct digestion with KOH, and after adsorption of the unsaponifiable residue the 7-dehydrosterol was in this instance eluted by light petroleum containing 20% of ether. The absorption maximum of the crude extract was at 272 mμ., but after chromatography the maximum was transferred to 282 mμ., with changes in the relative intensities of the other maxima to produce an absorption spectrum closely similar to those of the pure 7-dehydrosterols. This change was obviously due to the removal of substances having unselective absorption increasing in intensity at the shorter wavelengths.

The location of 7-dehydrosterol in the sexual organs of the rat. Fig. 2 indicates the regions in the sexual organs of the adult male rat in which 7-dehydrosterol was detected. The highest concentration, as judged by measurements on the crude fat,

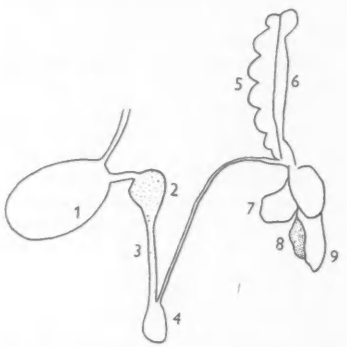

Fig. 2. Reproductive system of the male rat. The dotted areas indicate the positions at which the 7-dehydrosterol was detected. 1, testis. 2, caput epididymis. 3, corpus epididymis. 4, cauda epididymis. 5, seminal vesicle. 6, coagulating gland. 7, prostate. 8, preputial gland. 9, penis.

was found in the preputial glands; for piebald rats our richest extract had *E* (272 mμ.), 0·92. The next highest concentration was in the caput epididymis, with a maximum value for *E* (272 mμ.) of 0·22 for a similar crude preparation, and the corpus epididymis followed with *E* only 0·087. No absorption indicative of 7-dehydrosterol was observed in the testes, cauda epididymis, prostates, seminal vesicles or coagulating glands. In rats only 50 days old, which were still sexually immature, no selective absorption was found even in extracts of the caput epididymis.

In mature female rats the bulbi vestibuli, which appear to resemble the preputial glands of the male in form and function, contained about the same amount of 7-dehydrosterol, with *E*, 0·74. An extract was also made from the material which could be squeezed out from the bulbi vestibuli, and the spectrum of 7-dehydrosterol was again found with *E*, 1·31. The same methods of extraction failed to show any 7-dehydrosterol in the ovaries, oviducts and uteri. In an extract from one collection of vaginal plugs, the small lumps of cheese-like material which are left in the vagina after copulation, an absorption band was found at 268 mμ. and inflexions at 297, 278 and 258 mμ. This spectrum was consistent with that of 7-dehydrosterol displaced towards shorter wavelengths by superimposed general absorption. In an extract from another collection of plugs, bands or inflexions were again found not far removed from the positions characteristic of 7-dehydrosterol at 294, 280, 269 and 264 mμ. Additional bands of unknown significance, however, were also seen at 315 and 300 mμ., with inflexions at 348 and 320 mμ.

In order to find out whether similar intense selective absorption could be observed in a site recognized as being rich in cholesterol, we examined an extract made from rats' brain. When our experimental procedure was applied in the usual way no selective absorption was observed.

The effect of diet and the breed of the rat. When the caput epididymis of rats which were deficient in vitamin A was irradiated it did not show the faint-yellow fluorescence which was the starting point of these studies. Spectrophotometric comparisons between crude extracts made from the caput epididymis of rats deficient and adequate in vitamin A, however, indicated the presence of about equal amounts of 7-dehydrosterol. Thus for the deficient animals *E* (272 mμ.) was 0·19, as compared with 0·20 after supplements of 3 i.u. daily had been given and 0·22 after 320 i.u. daily for 15 days. In animals which had been kept on an adequate stock diet *E* was 0·19. For rats deficient in vitamin D, the one value recorded for *E* was 0·11, but since the rats were much younger than in the other groups the significance of this finding is questionable.

Dried brewer's yeast, which is rich in ergosterol, is a component of the basal diet used in this laboratory for inducing vitamin A deficiency, and it is also sometimes given to our stock animals. Although it is generally held that ergosterol is not absorbed into the tissues it was considered advisable to examine organs from wild brown rats, in whose diet yeast would be an unlikely constituent. Absorption characteristic of 7-dehydrosterol was again found, with *E* (272 mμ.), 0·18 for the caput epididymis and 1·54 for the preputial glands. These observations also indicated that the concentration of 7-dehydrosterols in these sites is not confined to one breed of rat.

The antirachitic activity of an irradiated extract of rats' preputial glands. The apparently high concentration of 7-dehydrosterol in the preputial glands suggested that extracts after a brief ultraviolet irradiation should be strongly antirachitic. In order to test this inference, the glands of forty normal rats, weighing 6·86 g., were treated with ethanolic KOH. The crude unsaponifiable residue weighed 0·35 g. and *E* (282 mμ.), calculated on the original tissue, was 0·61. On the basis of $E^{1\%}_{1\,cm.} = 310$ (Morton, 1942) for the pure substance, this was equivalent to a maximum of about 13·5 mg. of 7-dehydrosterol. By chromatography *E* was decreased to 0·20, equivalent to a total yield of only about 4·4 mg. of the sterol. The partly purified material was dissolved in ether and three portions, each containing about 1·3 mg. of 7-dehydrosterol, were irradiated for periods of 2, 3·5 and 5 min. at a distance of about 5 cm. from a large Hanovia ultraviolet lamp. Spectrophotometric examination of each solution indicated that this treatment caused loss of the bands at 262 and 294 mμ., but since no band appeared at 265 mμ. it was obvious that the efficiency of the formation of vitamin D was very low. Finally, the ether was evaporated, and the residue from each portion was dissolved in arachis oil for testing upon rachitic rats by the radiographic method (Bourdillon, Bruce, Fischmann & Webster, 1931).

During a curative period of 8 days groups of four animals each were given the irradiated products, in total doses corresponding to about 0·14 mg. of 7-dehydrosterol. Without allowing for losses during manipulation each treated rat received the product made from three of the original glands. In every animal advanced healing of the rickets was observed, corresponding to numbers 6–8 on the radiographic scale of healing (Bourdillon *et al.* 1931). In four rats which were dosed only with arachis oil the severity of the rickets remained unaffected, with a scale number of zero.

Organs from other animals. The extension of our observations to other animals was sometimes handicapped by difficulties in recognizing the parts of the genital system which corresponded to those already studied in the rat; in particular it was difficult to distinguish between the three regions of the epididymis.

In a human epididymis there was no evidence of 7-dehydrosterol, but bands at 278 and 284 mμ. were found in the part taken to be the cauda. The testis had no selective absorption in the ultraviolet, but contained carotene and lycopene. In the epididymis of the bull there was again no evidence of 7-dehydrosterol, but extracts from both the caput and the cauda had a band at 260 mμ. No selective absorption was found in the caput epididymis in the guinea pig, rabbit or ram, in glands in the rabbit resembling the rats' preputial glands, or in the preen gland of the duck.

DISCUSSION

Localized sites for the formation of 7-dehydrosterol. Glover *et al.* (1952) have studied the equilibrium between cholesterol and 7-dehydrocholesterol in the intestines of the guinea pig, and have shown that when cholesterol is administered the concentration of 7-dehydrocholesterol is greatly increased. It is clear that the desaturation is readily effected, presumably by the enzyme system which these workers postulate. Our own evidence on the distribution of 7-dehydrosterol in the sexual organs of the rat suggests further that its formation may be highly localized, and may take place in sites which are remote both from the route of absorption of dietary sterols and from the concentrations of cholesterol in the brain and nerves.

Thus the caput and corpus epididymis, which are rich in 7-dehydrosterol, are connected in one direction with the testes and in the other with the cauda epididymis, in neither of which could substantial amounts of 7-dehydrosterol be demonstrated. Similarly, isolated concentrations of the sterol occur in the preputial glands and bulbi vestibuli, but these glands differ from the epididymis in being associated with the genital system only in so far as their ducts are directed externally towards the openings of the penis and vagina, respectively. Another detached locus for the formation of 7-dehydrosterol has already been found by Morton & Rosen (1949) in the ovaries of the frog.

The extent to which 7-dehydrosterol was concentrated in the sex organs of our rats may be appreciated by comparing our values with those found by Glover *et al.* (1952) for the intestinal tissues of their guinea pigs, which were considered to be especially rich in 7-dehydrosterol. In the caput epididymis and preputial glands of the piebald rat our extinction coefficients observed on the crude extract, and roughly corrected for irrelevant absorption, indicated concentrations of 0·4 mg. and 2 mg. of 7-dehydrosterol/g. of wet tissue, respectively. In comparison, Glover *et al.* (1952) found the intestines of normally nourished guinea pigs to contain only 0·1–0·4 mg./g.; very much increased levels of 7-dehydrosterol were found in animals which had been given massive doses of cholesterol.

7-Dehydrosterol and the sex functions. Evidence suggesting a function for the high concentrations of 7-dehydrosterol in the genital system of the rat is scanty, but it is perhaps significant that we found indications of its presence in the plugs of clotted semen formed in the vagina after copulation. In rabbits which have been ovariectomized after fertilization, Feyel Cabanes (1949) has claimed that the administration of 7-dehydrocholesterol reduces the dose of progesterone which is necessary to maintain gestation.

Preputial exudate as a source of provitamin D. Hou (1930) reported that the material secreted by the anal preen glands of birds contains provitamin D. It was claimed that the bird distributes the secretion over its feathers with its beak, and is protected from rickets by ingesting some of the same material, after exposure to sunlight, during subsequent preenings. As yet we have no evidence that the rat imitates the bird by using the secretion of its preputial glands for grooming its fur, but it is clear that a rich source of provitamin would be available. Thus in each of two adult wild rats we found that the glands contained about 0·4 mg. of 7-dehydrosterol, which theoretically could yield 16 000 i.u. of vitamin D if efficiently converted. Although our experiments suggest that the preputial exudate could readily be activated by irradiation, the nocturnal habits of the rat raise doubts that a mechanism similar to that in the bird could be effective. Rosenberg (1953), moreover, has failed to confirm that the preen glands of geese, ducks and chickens contain provitamins, which have been detected in high concentrations in the feet.

Other animals. Our persistent failure to detect 7-dehydrosterol in the sex organs of animals other than the rat indicates that there must be at least minor differences in sterol metabolism between different species among the higher animals. Only single examinations were made on sex organs from man and the ox, which showed absorption bands not due to 7-dehydrosterol. Further research would be necessary to decide whether these bands are con-

sistently present, and if so whether they indicate important peculiarities in the sexual biochemistry of the animals concerned.

SUMMARY

1. A 7-dehydrosterol, presumably 7-dehydrocholesterol, was detected spectrophotometrically in high concentrations in the caput epididymis, corpus epididymis and preputial glands of male rats, and in the bulbi vestibuli of female rats. It could not be detected in other parts of the male or female genital systems.

2. The concentration of 7-dehydrosterol in its sites of deposition was not influenced by substituting a stock diet for an experimental diet, nor by varying the intake of vitamin A. About the same amounts of 7-dehydrosterol were found in wild brown rats as in tame piebald rats. No 7-dehydrosterol was detected in the rudimentary epididymides of sexually immature rats.

3. An irradiated extract from preputial glands had strong antirachitic activity.

4. No evidence of similar concentrations of 7-dehydrosterol was found in the male sex organs of the limited number of other animals investigated.

Our thanks are due to Dr L. J. Harris for his valuable criticism, to Dr D. B. Cater, Mr H. I. Field and Dr A. Walton for biological specimens, and to Miss P. J. Holder and Miss M. A. Tearle for technical assistance.

REFERENCES

Bourdillon, R. B., Bruce, H. M., Fischmann, C. & Webster, T. A. (1931). *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 158. H.M. Stationery Office.

Feyel Cabanes, T. (1949). *C. R. Soc. Biol., Paris*, **143**, 1454.

Glover, M., Glover, J. & Morton, R. A. (1952). *Biochem. J.* **51**, 1.

Hou, H. C. (1930). *Chin. J. Physiol.* **4**, 79, 93, 345.

Moore, T. & Ward, R. J. (1951). *Symp. biochem. Soc.* **7**, 33.

Moore, T. & Ward, R. J. (1952). *Biochem. J.* **52**, v.

Morton, R. A. (1942). *Vitamins*, 2nd ed., p. 27. London: Hilger.

Morton, R. A. & Rosen, D. G. (1949). *Biochem. J.* **45**, 612.

Rosenberg, H. R. (1953). *Arch. Biochem. Biophys.* **42**, 7.

The Activity of the Cytochrome System in Muscle and its Relation to Myoglobin

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Data secured during an investigation of factors affecting the percentage of myoglobin in muscle (Lawrie, 1950) had confirmed that a high concentration of this pigment is usually found in muscles of high physiological activity. This is of particular interest in view of the strong affinity of myoglobin for oxygen which enables it to act as an intramuscular oxygen store for the tissue's principal oxidizing catalysts, the cytochromes (Hill, 1936; Millikan, 1937). Keilin (1925) had early pointed out that all aerobic cells contain cytochromes, and that the concentration of cytochromes, as determined spectroscopically, is highest in the most actively respiring cells. Nevertheless, the concentration of cytochromes has been reported both as being higher (Fujita, Hata, Numata & Ajisaka, 1939) and lower (Hill, 1936) in myoglobin-rich or 'red' muscles than in the 'white' variety. It therefore appeared desirable to study the distribution of certain components of the cytochrome system in muscle and their relation to the content of myoglobin. A preliminary account of this work has already been published (Lawrie, 1952).

EXPERIMENTAL

An assessment of the cytochromes in muscle involves consideration of a series of components. Of these, only cytochrome *c* can be estimated quantitatively as protein. The determination of cytochrome *c* itself affords to a certain extent a measure of the cytochrome system as a whole, since its concentration in various tissues has been shown to follow closely the rate of O_2 uptake (Junowicz-Kocholaty & Hogness, 1939). However, it was the immediate relation of the cytochrome system to O_2 which was of interest in the present study. It was thus considered more appropriate to determine the system's power of catalysing the uptake of O_2 by assessing the activity of cytochrome oxidase (Keilin & Hartree, 1939; Slater, 1949*a*), which can be estimated by the rate of oxidation of ascorbic acid or *p*-phenylenediamine in the presence of excess cytochrome *c*. For the determination, the procedure of Slater (1949*b*) has been followed, as

described below. In addition, estimations were made of enzymic activity at succinate level. As a measure of succinic dehydrogenase, the rate of reduction of methylene blue in the presence of excess sodium succinate was employed. As a measure of succinic oxidase, the rate of O_2 uptake during the oxidation of sodium succinate in the presence of excess cytochrome *c* was employed. The heart-muscle preparation of Keilin & Hartree (1938, 1947) was used since it contains the complete cytochrome system including succinic dehydrogenase. It has been necessary to assume that strict adherence to their procedure would ensure the production of comparable enzyme-bearing particles in each preparation. Such an assumption seems reasonable in view of the similarity in activity found by Slater (1949*c*) in a large number of preparations of this kind. To relate enzyme activity in muscle preparations to the basis of whole muscle, it is necessary to assume further that high activity per unit weight of enzyme-bearing particles is reflected by a correspondingly high 'density' of such particles in the whole tissue. There is tentative support for this in the present work.

Preparation of enzyme suspensions from muscle. The method of Keilin & Hartree (1947) for heart-muscle preparations was employed for all the muscles examined. It was scaled down to handle relatively small amounts of muscle mince and was strictly standardized to allow comparison of the data secured. 80 g. mince, 25 g. acid-washed sand, and 160 ml. 0·02 M-phosphate buffer, pH 7·3, were ground together for 2 hr. The mixture was then diluted with a further 65 ml. of 0·02 M-phosphate buffer and centrifuged for 20 min. at 2000 rev./min. Thereafter the method and quantities followed the original. The activity of the resultant enzyme preparations was always measured within 20–36 hr. to minimize the effect of possible deterioration.

Estimation of the activities of cytochrome oxidase, the succinic-oxidase system and succinic dehydrogenase. Cytochrome oxidase was measured in Barcroft manometers, at 38–39°, by determining the rates of oxidation of neutralized ascorbic acid in 0·1 M-phosphate buffer, pH 7·3, and in the presence of known amounts of added cytochrome *c* (Slater, 1949*b*). By extrapolation the activity of cytochrome oxidase at infinite cytochrome *c* concentration could be calculated: this gave the maximum rate of O_2 uptake which the enzyme could catalyse under optimum conditions. The succinic-oxidase system was measured in Barcroft manometers, at 38–39°, by determining the rate of oxidation of sodium succinate in 0·18 M-phosphate buffer, pH 7·3, and in the presence of excess cytochrome *c* (Slater, 1949*c*). Succinic dehydrogenase was measured anaerobically at 37° using the Thunberg technique, by determining the time for 90% reduction of 0·00043 M-methylene blue in 0·18 M-phosphate buffer, at pH 7·3, and in the presence of excess sodium succinate (cf. Tsou, 1951).

The activity of these three enzyme systems was expressed as µl. O_2 taken up/mg. dry, fat-free weight of enzyme solids/hr. The Q_{O_2} values for succinic dehydrogenase were measured at suboptimal levels of methylene blue, since this dye poisons the enzyme in high concentrations (Slater, 1949*c*).

The dry, fat-free weight of the enzyme preparations was determined as described by Slater (1949*c*). Cytochrome *c* (containing 0·34% iron) and iron were determined by the methods of Keilin & Hartree (1945). Myoglobin was determined by a method previously described by the author (Lawrie, 1950). It was prepared for manometric work as metmyoglobin (Lawrie, 1951), since the ferrous forms of the pigment readily autoxidize.

Phosphate buffers were prepared from A.R. Na_2HPO_4 and NaH_2PO_4, $2H_2O$.

L-Ascorbic acid, B.P., and sodium succinate, laboratory reagent grade, were used; distilled water was employed for all solutions.

RESULTS

Variation of enzymic activities with age of animals

The general form of Figs. 1–3 shows that up to an age of 2 yr., the enzymic activities of cytochrome oxidase, the succinic-oxidase system and succinic

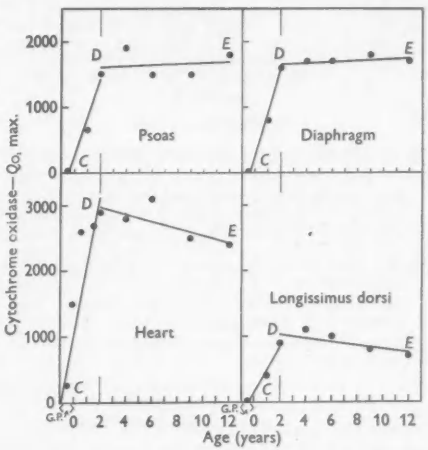

Fig. 1. The effect of the age of the animal on the cytochrome-oxidase activity of enzyme preparations from various horse muscles. G.P. = gestation period; *CD* = linear regression, 0–2 yr.; *DE* = linear regression, 2–12 yr. Cytochrome oxidase $-Q_{O_2\ max.}$ = µl. O_2 uptake/mg. fat-free dry enzyme solids/hr., at infinite cytochrome-*c* concentration. Experimental conditions (Slater, 1949*b*): enzyme preparation diluted tenfold with 0·10 M-phosphate buffer, pH 7·3, 0·2 ml.; ascorbic acid (50 mg./ml., neutral), 0·3 ml.; cytochrome *c*, $6{\cdot}7 \times 10^{-4}$ M in 0·5% NaCl, 0·1–0·3 ml.; phosphate buffer, 0·10 M, pH 7·3, 2 ml.; water, 0·5 ml.; total vol., 3·3 ml.

dehydrogenase, respectively, increase swiftly in the following muscles of horse: psoas, diaphragm, heart and longissimus dorsi (l. dorsi); thereafter in mature animals, all three enzymic activities appear to change relatively little. The data have been treated statistically in Table 1, from which it may be seen that the initial rates of increase of the three enzymic activities in preparations from the four muscles represent significant correlations with age up to 2 yr.: subsequent changes are insignificant.

Another interesting feature of Figs. 1–3 is the fact that early in the gestation period when no enzymic activity is exhibited in psoas, l. dorsi and

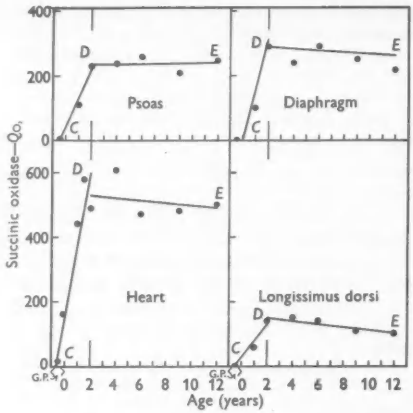

Fig. 2.

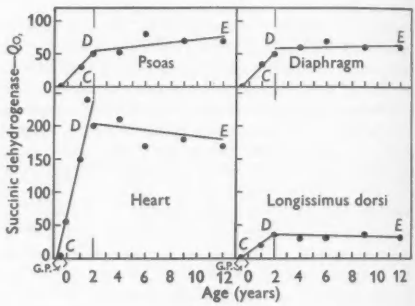

Fig. 3.

Fig. 2. The effect of the age of the animal on the activity of the succinic-oxidase system in enzyme preparations from various horse muscles. G.P. = gestation period; *CD* = linear regression, 0–2 yr.; *DE* = linear regression, 2–12 yr. $Q_{O_2} = \mu l.\ O_2$ taken up/mg. fat-free dry enzyme solids/hr. Experimental conditions (Slater, 1949*c*): enzyme preparation diluted fivefold with 0·18 M-phosphate buffer, pH 7·3, 0·2 ml.; 0·4 M-sodium succinate, 0·2 ml.; cytochrome *c*, $6{\cdot}7 \times 10^{-4}$ M in 0·5% NaCl, 0·2 ml.; phosphate buffer, 0·18 M, pH 7·3, 2·7 ml.; total vol, 3·3 ml.

Fig. 3. The effect of the age of the animal on succinic-dehydrogenase activity in enzyme preparations from various horse muscles. G.P. = gestation period; *CD* = linear regression, 0–2 yr.; *DE* = linear regression, 2–12 yr. $Q_{O_2} = \mu l.\ O_2$ taken up/mg. fat-free dry enzyme solids/hr. Experimental conditions (cf. Tsou, 1951): enzyme preparation diluted fivefold with 0·18 M-phosphate buffer, pH 7·3, 0·5 ml.; 0·4 M-sodium succinate, 0·2 ml.; 0·005 M-methylene blue, 0·3 ml.; phosphate buffer, 0·18 M, pH 7·3, 2·5 ml.; total vol., 3·5 ml. Time for 90% reduction measured.

Table 1. *Statistical analysis of data in Figs.* 1–3

(*CD* = linear regression 0–2 yr. *DE* = linear regression 2–12 yr.)

Muscle	Linear regression coefficients *CD*	Significance	*DE*	Significance
Cytochrome oxidase (Fig. 1)				
Heart	+1001·8957	+	−55·6962	Nil
L. dorsi	+ 360·5463	+	−30·0633	
Diaphragm	+ 651·8285	+	+11·0759	
Psoas	+ 605·9563	+	+ 9·1772	
Succinic-oxidase system (Fig. 2)				
Heart	+ 218·0095	+	− 4·2722	Nil
L. dorsi	+ 57·3399	+	− 4·9684	
Diaphragm	+ 117·0629	+	− 6·2975	
Psoas	+ 94·7237	+	+ 0·2532	
Succinic dehydrogenase (Fig. 3)				
Heart	+ 90·6398	+	− 3·4494	Nil
L. dorsi	+ 14·5645	+	− 0·1741	
Diaphragm	+ 21·1007	+	+ 0·6329	
Psoas	+ 20·8713	+	+ 2·1835	

+, Significant at 5% probability level.

Table 2. *Enzymic activities in preparations from the muscles of adult horses*

($Q_{O_2} = \mu l.\ O_2$/mg. dry, fat-free wt. enzyme solids/hr.)

Muscle	(*a*) Succinic-oxidase system	(*b*) Cytochrome oxidase	(*c*) Succinic dehydrogenase	Ratios *b*/*a*	Ratios *c*/*a*
Heart	510	2700	190	5·3	0·37
Diaphragm	260	1700	60	6·5	0·23
Psoas	240	1600	60	6·6	0·25
L. dorsi	130	900	30	6·9	0·23

diaphragm, the activities of the succinic-oxidase system, succinic dehydrogenase, and especially cytochrome oxidase, are appreciable in the heart. In Table 2 are given values for the activities of the three enzyme systems in preparations from the four muscles studied, averaged over the period 2–12 yr.,

Table 3. *Enzymic activities in preparations from horse heart during early phase of growth, as % average adult value*

Age	Cytochrome oxidase	Succinic-oxidase system	Succinic dehydrogenase
5 months before birth	10	3	3
6 weeks before birth	56	31	29
1 yr. after birth	96	86	79

i.e. in the adult animal. It is obvious that the three enzymic activities are considerably higher in the heart than in the skeletal muscles. While the ratios of the average activities of cytochrome oxidase, succinic oxidase, and succinic dehydrogenase, are of the same order in the two skeletal muscles and the diaphragm, they are different in the heart. Generally, the average values for heart are lower than those obtained by Slater (1949*c*), who has reported a Q_{O_2} of 3200, 625 and 240 for cytochrome oxidase, the succinic-oxidase system and succinic dehydrogenase respectively. Values of this order have been, however, observed in individual cases.

When the enzymic activities for horse heart obtained from animals less than 2 yr. old are expressed as a percentage of the corresponding average values for preparations from adult animals (cf. Table 3), it may be seen that cytochrome-oxidase activity increases initially at a markedly higher rate than succinic dehydrogenase and the succinic-oxidase system. This difference becomes less marked after 1 yr. from birth. The data in Table 1 (column *CD*) show that the rates of increase in the activity of the three enzyme systems are highest in heart, intermediate in diaphragm and psoas, and least in l. dorsi. When, however, these rates are expressed not in terms of Q_{O_2} units but as percentages of the final adult values, a high degree of similarity is revealed. This has been done in Table 4 which also gives the corresponding values for myoglobin, calculated from data given in a previous paper (Lawrie, 1950). It can be seen from this table that the high rates of increase of enzymic activities in the period 0–2 yr., and the very slow rates of change in the period 2–12 yr., are not significantly different from the corresponding changes in the percentage of myoglobin.

Species variations

In Table 5 the Q_{O_2} values are given for cytochrome oxidase, the succinic-oxidase system and succinic dehydrogenase in muscle preparations from a variety of species. For comparative purposes the corresponding data for the percentage of myoglobin in whole muscle have been included. In general, the activities of the three enzyme systems decrease together, but succinic dehydrogenase does so to a proportionately greater extent. It may be noticed, also, that myoglobin concentrations in the whole muscle tend to follow cytochrome-oxidase activities in the enzyme preparations. In this respect, however, whale-psoas, horse-heart and pigeon-pectoral muscles are striking exceptions. In Fig. 4, the percentages of myoglobin in the psoas muscles of different species have been plotted against cytochrome-oxidase activities in the corresponding enzyme preparations. The values, except those for elephant and blue whale, lie on a sigmoidal curve.

Table 4. *Yearly increase in enzymic activities and myoglobin concentration of muscles in young horses expressed as percentage of the average values in adult animal*

Muscle	Cytochrome oxidase	Succinic-oxidase system	Succinic dehydrogenase	Myoglobin
(*a*) 0–2 yr.				
Heart	+37·0	+42·7	+47·7	+38·9
Diaphragm	+38·4	+45·0	+35·0	+40·1
Psoas	+37·9	+40·0	+34·7	+42·8
L. dorsi	+40·0	+44·1	+46·7	+37·3
Mean	**+38·3**	**+42·9**	**+41·0**	**+39·8**
S.E.	± 1·8	± 2·2	± 7·1	± 2·4
(*b*) 2–12 yr.				
Heart	− 2·1	− 0·8	− 1·8	+ 0·7
Diaphragm	+ 0·7	− 2·4	+ 1·1	+ 3·6
Psoas	+ 0·6	+ 0·1	+ 3·6	+ 2·1
L. dorsi	− 3·3	− 3·8	− 0·6	+ 1·5
Mean	**− 1·0**	**− 1·7**	**+ 0·6**	**+ 2·0**
S.E.	± 1·8	± 1·7	± 2·7	± 1·2

Table 5. *Enzymic activities in muscle preparations from various species and myoglobin concentrations in corresponding whole muscle*

Species	Muscle	Q_{O_2} Cytochrome oxidase	Q_{O_2} Succinic-oxidase system	Q_{O_2} Succinic dehydrogenase	Myoglobin (% wet wt.)
Horse	Heart	2700	510	190	0·33
Pigeon	Pectoral	2300	260	50	0·22
Manx shearwater	Pectoral	1800	230	50	0·69
Horse	Diaphragm	1700	260	60	0·61
Horse	Psoas	1600	240	60	0·71
Ox	Psoas	1200	160	40	0·60
Pig	Psoas	1000	130	20	0·43
Sheep	Psoas	950	180	30	0·35
Horse	L. dorsi	900	130	30	0·47
Elephant	Psoas	800	140	30	0·46
Hare	Psoas	650	110	30	0·16
Blue whale	Psoas	600	80	20	0·84
Rabbit	Psoas	250	60	10	0·02

Anomalous enzymic activity in avian breast muscle preparations

The content of cytochrome *c* in pigeon-breast muscle is about twice as high as in horse heart (Junowicz-Kocholaty & Hogness, 1939). In view of the relationship between the content of cytochrome *c* and the rate of oxygen uptake by the tissues, it might have been expected that the enzymic activity of the avian pectoral muscle should also be high. Spectroscopic examination of the breast muscles from willow warblers killed immediately after their migratory flight from southern latitudes and from Manx shearwaters (birds which fly for great distances without rest) certainly revealed a concentration of cytochrome *c* which was high even in comparison with pigeon. However, as can be seen from Table 5, the enzymic activities of preparations from pigeon and Manx shearwater pectoral muscles are lower than those from horse heart. This was even more surprising in view of the well-known capacity of minced pigeon-breast muscle to sustain a rate of oxygen uptake considerably higher than that of horse heart (cf. Krebs & Johnson, 1948). A more detailed comparison of enzymic preparation from horse heart and from pigeon breast was therefore made.

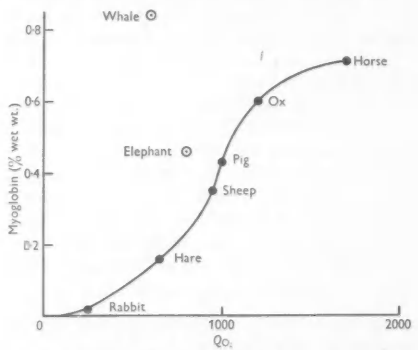

Fig. 4. The relation between myoglobin concentration in psoas muscle from the adults of various animals and cytochrome-oxidase activity (Q_{O_2}) in the corresponding enzyme preparations. Q_{O_2} = calculated μl. O_2 uptake/mg. fat-free dry enzyme solids/hr., at infinite cytochrome-*c* concentration (Slater, 1949*b*). Experimental conditions as in Fig. 1.

80 g. mince were used for each preparation. A summary of the data obtained is given in Table 6. The term 'intermediate layers' (I.L.) refers to gel-like material which came down in the centrifuge at 2000 rev./min. It was clearly quite distinct from the underlying fibrous mass of sand and connective tissue, etc., which is normally obtained at this stage, and from the enzyme colloid, which remained in suspension.

It is evident that there is a profound difference between preparations from the horse-heart and pigeon-breast muscle. The concentration of I.L. is 10 times higher in pigeon-breast muscle than in an equivalent weight of horse heart. As a result, although the I.L. has low enzymic activity, it accounts for a considerable proportion of the total oxygen uptake in the pigeon-breast muscle. Indeed, when its contributon is added to that of the corresponding normal preparation, the oxygen uptake of pigeon breast (40 900) is seen to exceed that of horse heart (30 700) by 30 %.

The possibility was investigated that the I.L. added to a succinic-oxidase preparation may exert a specific effect on the enzymic activity. However, as can be seen from Table 7, both in the absence and in the presence of cytochrome *c*, the I.L. has only an additive effect; even when incubated with pigeon-breast muscle preparation, it does not enhance the activity.

Table 6. *Comparative data on cytochrome-oxidase activity in muscle preparations from horse heart and pigeon breast*

Preparation	Cytochrome oxidase Q_{O_2}	Vol. prep. (ml.)	Mg. dry fat-free wt./ml.	Calculated O_2 uptake in whole tissue (μl./g./hr.)	
Horse heart:					
Normal	2500	30	30	28 100	30 700
Intermediate layer	700	10	30	2 600	
Pigeon breast:					
Normal	2300	20	28	16 100	40 900
Intermediate layer	640	100	31	24 800	

Table 7. *Succinic-oxidase activity of pigeon-breast muscle (PBMP)*

	Contents of right-hand flasks of manometers Volume (ml.)				
PBMP	Cytochrome (6×10^{-4} M)	Sodium succinate, 0·4 M	Phosphate buffer, 0·18 M	PBMP I.L.	O_2 uptake (μl./hr.)
0·2	0·2	0·2	2·7	—	480
—	0·2	0·2	2·7	0·2	160
0·2	0·2	0·2	2·5	0·2	600
0·2	—	0·2	2·7	—	370
—	—	0·2	2·7	0·2	110
0·2	—	0·2	2·5	0·2	470
0·2*	0·2	0·2	2·7	—	290
0·1†	0·2	0·2	2·7	0·1†	150
—	0·2	0·2	2·7	0·2*	65

* Incubated at 37° for 2 hr.
† Equal vols. I.L. and PBMP incubated together at 37° for 2 hr.; 0·2 ml. mixture used.

DISCUSSION

Before considering the principal results of this investigation, it is first desirable to assess the implications regarding technique arising from the discovery of a large quantity of I.L. gel in pigeon-breast muscle. With enzyme preparations from all sources, as has been mentioned above, procedures were identical. It is therefore most unlikely that activities differed merely through casual admixture of more or less I.L. gel material having low Q_{O_2} and arising from curtailment or prolongation of centrifuging or grinding operations outside normal limits. Had low enzymic activities in the muscle preparations been due to incomplete centrifugal removal of such gel material, the corresponding content of 'dry, fat-free enzyme solids', calculated per gram of whole tissue, would have been relatively high. In fact, low enzymic activity in preparations corresponded to a low 'density' of enzyme solids in the whole tissue and vice versa (Table 8).

The present study shows that variations in the activities of the cytochrome system are directly paralleled by corresponding variations in the percentages of myoglobin in the whole tissue. As may be seen from Tables 4 and 5, this parallelism extends over muscles differing in age, type and species. At the beginning of the century, Batelli & Stern (1907, 1912) had shown that, in general, the overall oxygen uptake and, in particular, the oxidation of *p*-phenylenediamine and succinate by washed muscle, was greater in 'red' than in the 'white' variety. Later Ahlgren (1921), besides confirming this work, showed that 'red' muscle had a greater capacity for reducing methylene blue. Nevertheless, there has been hitherto no attempt to carry out a co-ordinated study on myoglobin and the cytochrome system.

Table 8. *Relation of cytochrome-oxidase activity to calculated 'density' of enzyme solids in whole tissue*

Muscle preparation	Q_{O_2} (μl./mg. dry, fat-free wt./hr.)	Mg. fat-free dry enzyme solids/g. wet tissue
Horse heart	2700	12
Pigeon pectoral	2300	11
Horse psoas	1600	8
Horse diaphragm	1700	7
Horse l. dorsi	900	3
Whale psoas	600	1
Rabbit psoas	250	1

A particularly interesting example of the cytochrome-myoglobin relationship is shown by the data for the closely related species rabbit and hare. Low myoglobin levels in the muscles of the former

have been attributed to infrequent muscle usage (Hammond, 1932). It may now be seen that the activity of the cytochrome system is also low in rabbit psoas, whereas in that of the hare, an animal capable of enduring prolonged muscular exertion, both myoglobin and the activities of the cytochrome enzymes are high.

Striking non-conformity to the sigmoid myoglobin-cytochrome oxidase relationship (Fig. 4) is shown by the blue whale psoas muscle. The content of myoglobin in this muscle is approximately the same as that of the horse psoas, yet enzyme preparations made from it are only one-third as active. Moreover, from the data in Table 8, it can be calculated that on a whole tissue basis the difference is even greater: thus the store of oxygen represented by 0·7–0·8 % oxygenated myoglobin will last for about 20 times as long in whale psoas as in the corresponding muscle of the horse under conditions of maximum activity. Although 48 hr. elapsed between harpooning (off the Island of Harris) and the experimental determinations, the muscle samples were kept in an insulated container at a temperature just above the freezing point during the greater part of this time, and it is considered unlikely that any major deterioration of enzymic activity occurred. In any case the enzyme solids were found to be very low, which is in line with the low activity (Table 8). It may be pointed out that the normal duration of the dive in the sperm whale is about four times as long as that in the blue whale (Irving, 1939); and its muscles have a myoglobin content 5 times as great as those of the latter (Case, 1953). The other exception to the relationship shown in Fig. 4 is the elephant. However, the animal from which the samples were obtained had been in captivity for 10 yr., and the enforced inactivity may have had an adverse effect on its musculature. Such conditions certainly alter normal myoglobin levels (Lawrie, 1953). According to Krogh (1922) active muscles have a greater degree of vascularization, and thus a more plentiful blood supply, than those used less frequently. Differences of vascularity may exist between the psoas muscles of the different animals, and may account for the sigmoidal shape of the curve relating myoglobin to cytochrome-oxidase activity.

The importance of muscle function, in the widest sense, in determining the activity of the oxygen-utilizing enzyme systems is emphasized by the elaboration of the latter in heart muscle early in gestation when this organ probably represents the only muscle in action (Figs. 1–3). In adult life (Table 2) the heart is still by far the most continuously used muscle. This is reflected by a higher enzymic activity in heart than in skeletal muscles. Of the muscles studied the psoas and diaphragm are next in functional activity to the heart and the enzymic activities are found to be correspondingly intermediate between those of heart and l. dorsi.

The low cytochrome-oxidase activity of enzyme preparations from pigeon-breast muscle in relation to those from horse heart (Table 5) was at first difficult to reconcile with reports that minced horse heart had a considerably lower capacity for oxygen uptake than minced pigeon-breast muscle. The contribution of the large volume of I.L. gel obtained from the latter to the overall oxygen uptake of this tissue (calculated on a cytochrome oxidase basis) appears largely to explain the discrepancy. If the existence of a large volume of I.L. material in pigeon-breast mucle be accepted as a genuine phenomenon, one might regard the gel as an aggregate of tissue particles considerably larger in size than those of the highly colloidal suspension normally employed in Q_{O_2} studies. Although exhibiting little activity itself, and apparently ineffective in enhancing the oxygen uptake of the colloidal suspension *in vitro*, it may well represent a tissue structure which is especially necessary *in vivo* for maximum enzymic activity in the unusually vigorous pigeon-breast muscle.

Although, in general, the activity of the succinic dehydrogenase-cytochrome system in enzyme preparations has been shown to increase as the percentage of myoglobin in the whole muscle increases, the quantity of myoglobin in heart is disproportionately low as judged by this criterion. This would follow from the possession by this organ of a blood supply capable of constantly providing oxygen in amounts almost sufficient to meet the needs of its oxidase enzymes, high though these demands might be. Even so, it is particularly noteworthy that such myglobin as the heart does possess varies directly with the cytochrome enzymes in the developing animal in precisely the same way as in skeletal muscle (Table 4). This not only reconciles the divergent views in the literature, but also provides strong evidence that myoglobin does indeed function as an oxygen store for the tissue-oxidizing enzymes.

SUMMARY

1. The activities of succinic dehydrogenase, the succinic-oxidase system and cytochrome oxidase were determined in enzyme preparations from a variety of muscles: the effects of age, type of muscle and species, were considered.

2. In preparations from heart, longissimus dorsi, diaphragm and psoas muscles of the horse the three enzymic activities increased rapidly up to the age of 2 yr. and thereafter were relatively constant.

3. In the adult animal the order of increasing enzymic activity was given by: longissimus dorsi, psoas, diaphragm and heart.

4. In enzyme preparations from the foetal horse heart the activity of cytochrome oxidase was appreciable when that of succinic dehydrogenase and the succinic-oxidase system was very low.

5. In muscle preparations having relatively high cytochrome-oxidase activity, and especially in those from horse heart, the activities of succinic dehydrogenase and of the succinic-oxidase system were proportionately greater than in those with low cytochrome-oxidase activity.

6. During the preparation of enzyme suspensions from pigeon-breast muscle, a large quantity of gel material was always obtained. This had low enzymic activity: its significance is discussed.

7. A significant direct correlation was found between the percentage of myoglobin in muscle and the activity of the corresponding enzyme preparation.

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REFERENCES

Ahlgren, G. (1921). *Skand. Arch. Physiol.* **41**, 1.
Batelli, M. F. & Stern, K. (1907). *J. Physiol. Path. gén.* **9**, 134.
Batelli, M. F. & Stern, K. (1912). *Biochem. Z.* **46**, 317.
Case, R. A. M. (1953). *D.S.I.R. Food Investign Bd Spec. Rep.* no. **58**.
Fujita, A., Hata, T., Numata, I. & Ajisaka, M. (1939). *Biochem. Z.* **301**, 376.
Hammond, J. (1932). *Growth and Development of Mutton Qualities in the Sheep*, 1st ed. London: Oliver and Boyd.
Hill, R. (1936). *Proc. Roy. Soc.* B, **120**, 472.
Irving, L. (1939). *Physiol. Rev.* **19**, 112.
Junowicz-Kocholaty, R. & Hogness, T. R. (1939). *J. biol. Chem.* **129**, 569.
Keilin, D. (1925). *Proc. Roy. Soc.* B, **98**, 312.
Keilin, D. & Hartree, E. F. (1938). *Proc. Roy. Soc.* B, **125**, 171.
Keilin, D. & Hartree, E. F. (1939). *Proc. Roy. Soc.* B, **127**, 167.
Keilin, D. & Hartree, E. F. (1945). *Biochem. J.* **39**, 289.
Keilin, D. & Hartree, E. F. (1947). *Biochem. J.* **41**, 503.
Krebs, H. A. & Johnson, W. A. (1948). *Tabul. biol., Hague*, **19**, (iii), 140.
Krogh, A. (1922). *The Anatomy and Physiology of Capillaries.* Yale University Press. Cited by G. Biörck (1949). *Acta med. scand.* **133**, Suppl. 226.
Lawrie, R. A. (1950). *J. agric. Sci.* **40**, 356.
Lawrie, R. A. (1951). *Nature, Lond.*, **167**, 802.
Lawrie, R. A. (1952). *Nature, Lond.*, **170**, 122.
Lawrie, R. A. (1953). *Nature, Lond.* (in the Press).
Millikan, G. A. (1937). *Proc. Roy. Soc.* B, **123**, 218.
Slater, E. C. (1949*a*). *Biochem. J.* **45**, 14.
Slater, E. C. (1949*b*). *Biochem. J.* **44**, 305.
Slater, E. C. (1949*c*). *Biochem. J.* **45**, 1.
Tsou, C. L. (1951). *Biochem. J.* **48**, lii.

The Relation of Energy-rich Phosphate in Muscle to Myoglobin and to Cytochrome-oxidase Activity

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In a previous communication (Lawrie, 1953) it has been shown that a statistically significant correlation exists in muscle between the percentage of myoglobin and the activity of the succinic dehydrogenase-cytochrome system. Myoglobin is the oxygen-storing pigment principally responsible for red coloration in muscle, the succinic dehydrogenase-cytochrome system catalyses intracellular oxygen uptake for energy production by the oxidation of metabolites. It is generally agreed (cf. Needham, 1942) that the main form in which energy can be used by muscle for mechanical work is as it is stored in the terminal energy-rich phosphate (~P: Lipmann, 1941) of adenosine triphosphate (ATP). It seemed reasonable to anticipate that the production of ~P might be related to the activity of the succinic de-

hydrogenase-cytochrome system and to the content of myoglobin. The results of some investigations on this problem are reported below. A preliminary account of the work has already been published (Lawrie, 1952).

EXPERIMENTAL

For several hours *post mortem*, there persists in excised muscle the dynamic equilibrium of metabolites which is characteristic of resting muscle *in vivo*. The maintenance of the muscle in its flexible, physiologically active condition becomes impossible only when the rate of synthesis of ATP falls below about 90% of the rate of its breakdown by myosin ATPase (Bendall, 1951). Rigor mortis then occurs. At this stage ATP is no longer available to prevent cross-bonding and, as a result, the contractile proteins, actin and myosin, become inextensible (Bate-Smith, 1948). This event occurs either when glycogen supplies become exhausted or when the accumulating lactic acid causes the pH to attain values suboptimal for ~P formation by glycolysis (Bendall, 1951). Therefore in the brief period between the death of the animal and the onset of rigor mortis in the excised muscle, conditions appeared to be favourable for the study of ~P synthesis. For any particular muscle, the difference between the aerobic and anaerobic rates should provide a measure of its power to produce ~P aerobically and this should bear a direct relation to the activity of the O_2-utilizing enzymes. This approach was adopted in the present work, replicate samples of each muscle being exposed to air and to N_2 during the period of 'surviving' metabolism.

Exposure of muscle samples to gas. Muscles were excised as soon as possible *post mortem* and connective tissue, fat and blood clots removed. The mince was exposed, in layers about 2 mm. thick, to N_2 or air at room temperature (15–20°) in vessels of about 500 ml. capacity which could be sealed at atmospheric pressure. The period of exposure was strictly standardized to run from the first to the seventh hour *post mortem*. The need to limit the mince to a thickness of 2 mm. was apparent from a consideration of the general permeability of such mince to O_2 where O_2-utilizing systems are still functioning. According to Hill (1929), this permeability is governed by an equation of the form: $d = \sqrt{(2CD/A)}$ where $C = O_2$ pressure in atm. at surface; D = diffusion coefficient of O_2 through the tissue expressed in ml. O_2 (s.t.p.), diffusing in 1 min. across 1 cm. when pressure gradient of O_2 is 1 atm./cm.^{2}; $A = O_2$ uptake of the tissue expressed in ml. O_2 (s.t.p.) consumed by 1 ml. of tissue in 1 min., and d = depth of penetration of O_2 when equilibrium is established between consumption and supply.

Brooks (1929), whilst studying the formation of muscle methaemoglobin (metmyoglobin) applied this equation to mammalian red muscle. On the assumption that where the characteristic purple of reduced myoglobin appears the ratio $\frac{\text{oxy-myoglobin}}{\text{reduced myoglobin}}$ is less than unity, he calculated that the bright-red layer of oxy-myoglobin on the outside of the muscle represented 84% of the total depth of O_2 penetration when O_2 demand and supply were in equilibrium. Thus the bright outer zone gave a good approximation to 'd' in Hill's formula. In the present study fresh minces from several distinct samples of various muscles were pressed between glass plates in air and the outer bright-red zones at the exposed edge of the minces measured at room temperature after 1 hr. (cf. Brooks, 1929). Values (mm.) were as follows: psoas, $0{\cdot}94 \pm 0{\cdot}12$; diaphragm, $0{\cdot}96 \pm 0{\cdot}13$; l. dorsi, $2{\cdot}48 \pm 0{\cdot}30$.

On the assumption that the tissue-oxidizing enzymes would be adequately supplied with gas when the ratio $\frac{\text{oxy-myoglobin}}{\text{reduced myoglobin}}$ could still be about unity as judged by Brooks's criterion, it seemed reasonable to suppose that a 2 mm. layer of mince exposed to gas on both sides represented suitable material for studying the effects of air or N_2 on the phosphorus metabolism of surviving muscle.

Preparation of muscle extracts. Muscle mince (1–2 g.) was plunged into 10 ml. 10% (w/v) trichloroacetic acid (TCA), held in the cup of a Marsh-Snow homogenizer (Marsh & Snow, 1950), and 'homogenized', with the motor running at full speed, for 1 min. 10 ml. of ice-cold water were now added, and the mixture filtered at 0° into a 50 ml. conical flask. The extract was neutralized to phenolphthalein and preserved at 0°.

Estimation of phosphorus. (1) Inorganic phosphate (IP) was determined by the method of Allen (1940); 95% of IP is estimated in 2·5 min. at 17° in the presence of Allen's amidol-perchloric acid reagent; this value was always corrected by the factor 100/95 to give the total IP. (2) Creatine phosphate phosphorus (CP-P) represents the phosphate, in excess of IP, liberated by Allen's reagent in 36 min. at 17° (Eggleton & Eggleton, 1929, as modified by Bendall, 1951). (3) Apparent adenosine triphosphate-phosphorus (ATP-P) was calculated on the basis that 1 N-HCl splits off two phosphate groups from ATP as well as the phosphate from creatine phosphate in 7 min. at 100° (Lohmann, 1931) so that phosphate estimated after 7 min. hydrolysis represents the sum of ATP-P + CP-P + IP. (4) Triose phosphate phosphorus (TrP-P) was estimated as phosphate liberated by N-NaOH in 20 min. at 17° (Meyerhof & Lohmann, 1934). (5) Hexose diphosphate (HDP-P) has been calculated on the assumption that 0·2 N-NaOH hydrolyses both hexose diphosphate and triose phosphate to inorganic phosphate in 3 min. at 100° (Marsh, 1951). (6) Total soluble phosphorus (TSP) was determined after digestion for 3 hr. with 60% (w/v) perchloric acid. (7) Energy-rich phosphate (~P) is considered to be the sum of true ATP-P and CP-P.

As 43% of triose phosphate is hydrolysed in 7 min. at 100° in the presence of N-HCl (Meyerhof & Lohmann, 1934) and under the same conditions 30% of hexose diphosphate is also hydrolysed (McLeod & Robison, 1933), the likely presence of these esters in the extracts will interfere with the determination of apparent ATP-P (3). Appropriate correction was therefore made in accordance with the procedure of Marsh (1951).

True ATP-P = (3–2) – 43% (4–1) – 30% (5–4) = apparent ATP-P – 43% TrP-P – 30% HDP-P.

Determination of ATPase activity. The method employed was that of Bendall (1952), and the determination was carried out at room temperature. Two 1 g. samples were weighed and thereafter ground in the 'homogenizer' for 1·5 min. in 10 ml. 0·16 M-KCl. They were then centrifuged at 3500 rev./min. for 5 min., the supernatant was discarded and the washing procedure was repeated. The fibre residue was then suspended in 5 ml. 0·16 M-KCl.

(i) To one sample were now added 15 ml. 5% (w/v) TCA. After regrinding, 0·5–1·0 mg. ATP-P in 0·5 ml. distilled

water was stirred in. The mixture was centrifuged, filtered through cotton wool and the volume measured (V).

(ii) To the other sample 0·5–1·0 mg. ATP-P was added in 0·5 ml. distilled water and after 1 min., 15 ml. 5% (w/v) TCA. The mixture was immediately reground. After centrifuging and filtering, the volume was adjusted to V. 1 ml. of each TCA extract was taken for determination of inorganic phosphate and the difference in colour development read on the Spekker absorptiometer by direct comparison of the two solutions after between 5 and 30 min.

ATPase activity (μg. P/min./g.) = absorptiometer reading (μg. P) × V.

Other experimental methods. Myoglobin concentration was determined by a method previously described (Lawrie, 1950) and cytochrome oxidase by the method of Slater (1949). pH was measured by glass electrode. For this purpose 0·5–1·0 g. samples of muscle were ground in 10 ml. 0·005 M-sodium iodoacetate (Bate-Smith & Bendall, 1947).

Reagents. A.R. grade chemicals were employed with the exception of sodium iodoacetate, and of ATP, which was prepared by Mr J. R. Bendall, to whom the author is indebted for a quantity of this substance.

RESULTS

In Fig. 1 the rates of aerobic ~P fall in minced psoas muscles from horses of various ages have been represented as fractions of the corresponding rates in nitrogen. In every case ~P was expressed as % of TSP. It may be seen that the relative power of aerobic conditions to diminish the rate of fall of ~P levels $\frac{\Delta \sim P/\Delta t \text{ (air)}}{\Delta \sim P/\Delta t \text{ (nitrogen)}}$ increases swiftly in the muscles of the young, developing animal, but remains constant after maturity. Statistical treatment reveals that the slope of *CD* (horses less than 2 yr. old) is significant at the 1% level and that of *DE* (horses 2–12 yr. old) is insignificant. Expressed as a percentage per year of the mean adult value, the rates of increase are given in Table 1, together with corresponding data on cytochrome-oxidase activity and myoglobin concentration (Lawrie, 1953). A striking correspondence between (i) the power to maintain ~P, (ii) cytochrome-oxidase activity, and (iii) the percentage of myoglobin, is then revealed.

Comparative data on oxygen-linked factors in muscles are summarized in Table 2; in each case the values are derived from not less than three adult animals. From the buffering power of muscles between pH 7 and pH 6 (approx. 6×10^{-5} equiv. acid/g. in horse and rabbit muscle: Bate-Smith, 1938) and the equivalence of 1 mole lactic acid to 1·5–2 moles ~P (Lipmann, 1941), a pH fall of 1·0 unit may be regarded as resynthesizing 3200 μg. ~P/g. muscle (Marsh, 1951). Other reactions taking place *post mortem* have little effect on pH in comparison with that due to lactic acid production (Bate-Smith & Bendall, 1949). The resynthesis of ~P corresponding to the observed fall of pH could thus be calculated. The observed rates of ~P fall are the values resultant from the combined processes of ATPase activity, resynthesis, esterification and depletion of the initial ~P stores. The latter were calculated roughly from ~P levels at 1 hr. *post mortem*, and the average rates of ~P fall between the 1st and 7th hours *post mortem*.

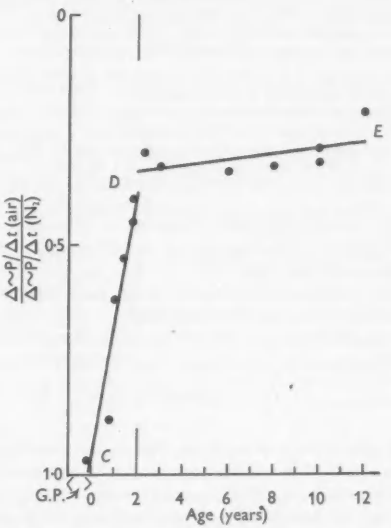

Fig. 1. The effect of animal age on the relative power of minced psoas muscle of the horse to maintain energy-rich phosphate levels during aerobic 'surviving' metabolism (1st–7th hr. *post mortem*). $\frac{\Delta \sim P/\Delta t \text{ (air)}}{\Delta \sim P/\Delta t \text{ (N}_2\text{)}}$ = ratio of rate of energy-rich phosphate fall in air (as % total soluble phosphorus/hour) to corresponding rate in N_2. G.P. = gestation period; *CD* = linear regression, 0–2 yr.: *DE* = linear regression, 2–12 yr.

Table 1. *Comparative rates of increase in (i) the capacity for aerobic ~P synthesis, (ii) cytochrome-oxidase activity and (iii) myoglobin concentration, in psoas muscles from immature and adult horses*

(The rates of increase are expressed as percentages per year of the average adult values for these three factors.)

Age	(i) $\frac{\Delta \sim P/\Delta t \text{ (air)}^*}{\Delta \sim P/\Delta t \text{ (N}_2\text{)}}$	(ii) Cytochrome oxidase (Q_{O_2})	(iii) Myoglobin (% wet wt.)
0–2 yr. (immature)	43·8	37·9	42·8
2–12 yr. (adult)	1·0	0·6	2·1

* The ratio of the rate of ~P fall in air to that in nitrogen between the 1st and 7th hour *post mortem* in minced muscle exposed to these gases: ~P determined in TCA extracts as % total soluble phosphorus (decrease in the ratio represents an increased capacity for aerobic ~P synthesis).

Table 2. *Comparative data on oxygen-linked factors in various muscles*

	Horse diaphragm	Horse psoas	Horse l. dorsi	Rabbit psoas
(*a*) Aerobic ~P metabolism in muscle mince: 1–7 hr. *post mortem*				
Rate of aerobic pH fall (units/hr.)	0·07	0·06	0·13	0·20
Equiv. ~P resynthesized (μg./hr./g.)	224	192	476	640
Obs. rate of aerobic ~P fall (μg./hr./g.)	15	15	80	172
Total gross ~P aerobic breakdown (μg./hr./g.)	239	207	556	812
~P esterified as HDP-P and TrP-P (μg./hr./g.)	12	5	12	6
Total net aerobic ~P breakdown (μg./hr./g.) (*x*)	227	202	544	806
(*b*) Anaerobic ~P metabolism in muscle mince: 1–7 hr. *post mortem*				
Rate of anaerobic pH fall (units/hr.)	0·13	0·11	0·19	0·23
Equiv. ~P resynthesized (μg./hr./g.)	416	372	608	736
Obs. rate anaerobic ~P fall (μg./hr./g.)	60	45	121	192
Total gross ~P anaerobic breakdown (μg./hr./g.)	476	417	729	928
~P esterified as HDP-P and TrP-P (μg./hr./g.)	17	1	10	7
Total net anaerobic ~P breakdown (μg./hr./g.) (*y*)	457	416	719	921
(*c*) ATPase activity in corresponding homogenates (μg. ~P/hr./g. tissue)*	5400	4800	9000	19200
(*d*) Relative capacity for aerobic ~P resynthesis expressed as $(y-x)/y$	0·51	0·51	0·24	0·12
(*e*) Cytochrome-oxidase activity (whole tissue basis) (μl. O_2/hr./g. tissue) (Lawrie, 1953)*	13600	11200	2700	250
(*f*) Calculated initial ~P level (μg./g. tissue)*	500	450	700	1100
(*g*) Myoglobin (% whole tissue) (Lawrie, 1952)*	0·61	0·71	0·47	0·02

* The corresponding values in horse heart are (*c*) 1800, (*e*) 32400, (*f*) 250 and (*g*) 0·33.

From Table 2 it appears that in the diaphragm, psoas and l. dorsi of the horse and in the psoas of the rabbit, both myoglobin and the activity of cytochrome oxidase, the enzyme principally responsible for oxygen uptake in muscle, bear a direct relation to the capacity for aerobic ~P resynthesis, which is here represented by the ratio

$$\left(\frac{\text{net anaerobic} - \text{net aerobic}}{\text{net anaerobic}}\right) \sim \text{P breakdown.}$$

It may also be noted that the percentage of myoglobin, and cytochrome-oxidase activity, bear an inverse relation to the initial ~P store, ATPase activity and to the total net aerobic and anaerobic breakdown in these muscles. Similarly, the very high cytochrome-oxidase activity in horse heart is associated with a low ~P store and low ATPase activity in the corresponding muscle homogenate; its myoglobin content is, however, unexpectedly low.

DISCUSSION

A direct correlation between the percentage of myoglobin, the activity of cytochrome oxidase and a high capacity for the aerobic formation of ~P is manifest not only between various types of muscle (Table 2) but also during the development of a selected muscle in the growing animal (Table 1). Greater restitutive powers in red muscle would be associated with sustained activity *in vivo*. In white (myoglobin-poor) muscle, on the other hand, there is a relative absence of respiratory activity, and a diminished capacity for energy-rich phosphate formation. Adenosine-triphosphatase activity is high, however, and there is a more abundant store of energy-rich phosphate. These are the prerequisites for a muscle dissipating energy at a high rate anaerobically, but functioning only in short bursts. There must be frequent rests for the restoration of energy-rich phosphate to the equilibrium level, for the respiratory system is too little developed to permit a high rate of synthesis during usage (even were an adequate oxygen supply available). The data for rabbit psoas, as a representative white muscle, and for horse l. dorsi, psoas and diaphragm, representing red muscles (Table 2) aline with the physiological observations that the rabbit is easily exhausted, whereas the horse is capable of sustaining considerable muscular effort over a long period. Horse heart which, save for a fraction of a second in each contraction, never rests, also well exemplifies this alinement. In such a muscle there can be little opportunity for the leisurely removal of lactic acid, of which relatively little is formed, and no oxygen debt is incurred (cf. Hill, Long & Lupton, 1924). There is a very small ~P reserve. The organ presumably relies on its high oxidative capacity, under aerobic *in vivo* conditions, for the adequate and efficient provision of ~P bonds. That the myoglobin value for the heart is disproportionately low in relation to its cytochrome-oxidase activity is probably due to the diminished need for an oxygen store in an organ provided with a profuse blood-borne oxygen supply.

The ATPase activities of the muscles investigated bear further consideration. It may be noted that the calculated ATPase activities (Table 2 (*b*)) for the minced skeletal muscles bear the same relation to one another as the values determined directly (Table 2 (*c*)). On an absolute basis, however, the minces have a much lower ATPase activity than that actually measured in the corresponding suspensions. In this connexion the 'turnover' of 921 μg. ~P/hr./g. in minced rabbit psoas may be compared with the value of 550 μg. ~P/hr./g. calculated for this same muscle when excised but unminced (Bate-Smith & Bendall, 1949). These findings would suggest that as the degree of organization increased, ATPase activity was suppressed, under conditions approximating to rest *in vivo*. As Mommaerts (1950) has calculated, however, the rate of ~P splitting must be as high as 2×10^6 μg. ~P/hr./g. in the musculature of intact animals after a burst of exercise to exhaustion. On this basis the *in vivo* ATPase activity could be several hundredfold greater than the degree of activity found even in the suspensions during the present study. It may thus be postulated either that myosin ATPase is much more labile in post-mortem conditions than has hitherto been supposed or that, *in vivo*, organization is such as to permit a vast increase in the activity of the enzyme as measured *in vitro*. Such a conclusion is by no means new in biological studies. Perry (1951) has indeed recently demonstrated that myosin ATPase is enhanced by myofibrillar organization.

In concluding, reference may be made to a preliminary report by Paul & Sperling (1951) suggesting that red muscles contain a greater concentration of mitochondria, and exhibit higher activity of the cyclophorase enzymes, which catalyse oxidative phosphorylation, than do the white varieties. Mitochondria have also been shown to be the histological entities in which resides the activity of the cytochrome system of enzymes (Schneider, 1947). There is thus independent evidence supporting and rationalizing the present demonstration of a parallelism in muscle between the percentage of myoglobin, the activity of cytochrome oxidase and a high capacity for aerobic synthesis of ~P.

SUMMARY

1. The relative rates of decrease of aerobic and anaerobic energy-rich phosphate during 'surviving' metabolism in muscle minces were used as a measure of the muscles' relative capacities to resynthesize energy-rich phosphate bonds in the presence of oxygen.

2. In horse psoas muscle this capacity was found to increase swiftly up to the age of 2 yr. and thereafter to remain at a relatively steady value. These changes bore a significant direct correlation with corresponding changes in myoglobin concentrations and in the activities of the succinic dehydrogenase-cytochrome system.

3. Investigations on various muscles of horse and rabbit showed that high myoglobin concentration (red muscle) was associated with a high capacity for energy-rich phosphate resynthesis aerobically, and high activities of the succinic dehydrogenase-cytochrome system; anaerobically it was associated with a low capacity to effect glycolysis, low adenosine triphosphatase activity and a low energy-rich phosphate store.

The Statistical Laboratory, Mathematics Department, analysed the data of Fig. 1 and those from which Table 1 was derived. I wish to thank Prof. D. Keilin, F.R.S., for his interest in this investigation. The work described in this communication was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

REFERENCES

Allen, R. J. L. (1940). *Biochem. J.* **34**, 856.
Bate-Smith, E. C. (1938). *J. Physiol.* **92**, 336.
Bate-Smith, E. C. (1948). *Advanc. Food Res.* **1**, 1.
Bate-Smith, E. C. & Bendall, J. R. (1947). *J. Physiol.* **106**, 177.
Bate-Smith, E. C. & Bendall, J. R. (1949). *J. Physiol.* **110**, 47.
Bendall, J. R. (1951). *J. Physiol.* **114**, 71.
Bendall, J. R. (1952). Personal communication.
Brooks, J. (1929). *Biochem. J.* **23**, 1391.
Eggleton, G. P. & Eggleton, P. (1929). *J. Physiol.* **68**, 193.
Hill, A. V. (1929). *Proc. Roy. Soc.* B, **104**, 39.
Hill, A. V., Long, C. N. H. & Lupton, H. (1924). *Proc. Roy. Soc.* B, **96**, 438.
Lawrie, R. A. (1950). *J. agric. Sci.* **40**, 356.
Lawrie, R. A. (1952). *Nature, Lond.*, **170**, 122.
Lawrie, R. A. (1953). *Biochem. J.* **55**, 298.
Lipmann, F. (1941). *Advanc. Enzymol.* **1**, 99.
Lohmann, K. (1931). *Biochem. Z.* **233**, 460.
McLeod, M. & Robison, R. (1933). *Biochem. J.* **27**, 286.
Marsh, B. B. (1951). Ph.D. Dissertation, Cambridge.
Marsh, B. B. & Snow, A. (1950). *J. Sci. Fd Agric.* **1**, 190.
Meyerhof, O. & Lohmann, K. (1934). *Biochem. Z.* **271**, 89.
Mommaerts, W. F. M. (1950). *Muscular Contraction.* New York: Interscience.
Needham, D. M. (1942). *Biochem. J.* **36**, 113.
Paul, M. H. & Sperling, E. (1951). Unpublished observations. Cited by D. E. Green (1951). *Biol. Rev.* **26**, 445.
Perry, S. V. (1951). *Biochem. J.* **48**, 257.
Schneider, W. C. (1947). *Cold Spr. Harb. Symp. quant. Biol.* **12**, 169.
Slater, E. C. (1949). *Biochem. J.* **44**, 305.

The Application of the Diacetyl Reaction to the Estimation of Creatine in Urine

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It has been shown (Ennor & Stocken, 1948) that sulphydryl or disulphide compounds interfere with the Barritt (1936) reaction as applied by Eggleton, Elsden & Gough (1943) for the determination of creatine in tissue extracts and that this interference can be overcome by the addition of *p*-chloromercuribenzoic acid to the reaction mixture. It thus became possible to estimate directly the creatine content of tissues regardless of their glutathione content. More recently this method has been applied to the determination of the phosphocreatine content of various organs (Ennor & Rosenberg, 1952). In the former publication (Ennor & Stocken, 1948) some advantage was claimed for the method in determining the creatine content of urine, but we subsequently found that the reaction was inhibited by compounds other than those of the sulphydryl type and that this inhibition could not be removed by the addition of *p*-chloromercuribenzoic acid. Raaflaub & Abelin (1950) have reported similar findings and have detailed a method for the estimation of creatine in urine in which the effect of inhibitors has been reduced by a dilution technique. The present report gives details of experiments designed to determine the nature of the inhibitors, the specificity of the reaction, and details of a method whereby creatine may be determined in urine. A preliminary report of this work has been given (Ennor & Stocken, 1949).

EXPERIMENTAL

Reagents

Creatine hydrate and creatinine. A.R. specimens were recrystallized according to Hunter (1928) and their purity checked by N analysis. All solutions were made up immediately prior to use.

Guanidine and guanidine derivatives. Most samples used were of A.R. quality and were used without further purification.

Diacetyl. A stock solution of approx. 1% was prepared from dimethylglyoxime as described by Walpole (1911) and diluted 1:20 for use.

1-*Naphthol.* Freshly prepared solutions were used throughout. A.R. quality was satisfactory provided that the 10% (w/v) solution in the stock alkali showed but a faint colour.

Stock alkali. NaOH (60 g.) and Na_2CO_3 (128 g.) were dissolved and made up to 1 l. in water.

p-*Chloromercuribenzoic acid.* This was synthesized as described by Whitmore & Woodward (1932). A 4×10^{-3} M solution was prepared by dissolving the requisite amount of the compound in the minimum volume of N-NaOH before making up to volume.

Development of method

In the earlier part of this work on model systems the conditions suggested by Eggleton *et al.* (1943) were observed but in experiments with urine it became desirable to increase the sensitivity of the method. This was accomplished by the use of higher 1-naphthol concentrations, the effect of which, on the extinction (*E*) using a constant amount of creatine, is shown in Fig. 1. In these experiments varying amounts of 1-naphthol were dissolved in 2·5 ml. of stock alkali and added to 10 μg. of creatine dissolved in water. 0·5 ml. of the diacetyl dilution was then added, the volume adjusted to 10 ml. by the addition of water and the solution thoroughly mixed. After standing for 20 min. for colour development the optical density was measured in a Hilger Spekker absorptiometer using an Ilford spectrum-green filter (no. 604).

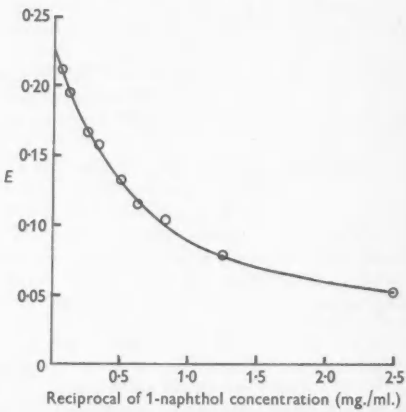

Fig. 1. Relationship between 1-naphthol concentration and *E* of 10 μg. of creatine as measured 20 min. after the addition of the reagents.

A plot of the extinction against the reciprocal of the 1-naphthol concentration (Fig. 1) indicates a limiting E greater than 0·2. A convenient practical concentration of 160 mg./10 ml. gives an E of about 0·2.

The estimation of creatine in the presence of creatinine

Model experiments. The estimation of creatine in urine, quite apart from the effect of accelerators or inhibitors which may be present, is complicated by the fact that small amounts of creatine are being determined in the presence of relatively large amounts of creatinine. This latter compound hydrolyses in the alkaline reaction mixture and yields creatine at a rate which is dependent upon temperature. The rate of this hydrolysis is such, at ordinary laboratory temperatures and at the pH of the reaction mixture, that the concentration of creatinine 20 min. after the preparation of the mixture is not significantly reduced and it follows therefore that creatine is formed at a constant rate. Since, however, the ratio of creatine to creatinine in urine is of the order μg./mg. it is clear that whilst the creatinine loss by hydrolysis may be insignificant the creatine gain is such that a serious error will be introduced unless this is taken into account. The colour density of a solution containing only creatine reaches a maximum at 20° in about 20 min. after which time there is a gradual decrease. In the case of a mixture of creatine and creatinine the colour density continues to increase. The density at 20 min. does not represent the simple sum of the colour due to the creatine initially present plus that due to the creatine produced by hydrolysis of the creatinine, because of the time lag in colour development and because of the continuing hydrolysis of creatinine. However, since the hydrolysis and colour development rates, though different, are constant, it should be possible by a simple extrapolation procedure to determine the initial creatine content. Such a method has in fact been proposed by Raaflaub & Abelin (1950), who have shown that an extrapolated curve obtained with creatinine passes through the origin. In experiments carried out in 1948 in Melbourne and in Oxford, although there was agreement on the principle of extrapolation and on the fact that an extrapolated curve did not pass through the origin, the point on the time axis (extrapolation time) at which this was cut by the extrapolated curve was the subject of controversy. It subsequently became clear that the disagreement was due to differences in temperature and the purity of the reagents, and we therefore undertook a careful purification of the reagents and a rigid control of the experimental conditions. The temperature was controlled either by carrying out the entire procedure in a constant temperature room, by withdrawing samples from a large bulk of reaction mixture maintained at constant temperature or by means of a jacketed absorption cell through which water at the requisite temperature was circulated.

The conversion of creatinine to creatine was followed by measurement of the colour density at various time intervals after the addition of the reagents. For this purpose three concentrations were employed, namely 200, 400 and 800 μg. of creatinine with and without 10 μg. creatine in a final volume of 10 ml.

It will be observed (Fig. 2) that with creatinine alone at 19° the extrapolation time is 5 min. and is independent of concentration. Curves (3–6) were obtained from a similar measurement of colour development but in the presence of added creatine (10 μg.). These also intersect at the 5 min. point but at a distance above the abscissa which is equal to the E of 10 μg. creatine alone. Additional experiments carried out at varying temperatures have led us to the conclusion that the extrapolation time is a function of temperature and is completely independent of creatinine concentration over the range 200–5600 μg./10 ml. The results obtained in these experiments are best shown by plotting the extrapolation time against the reaction temperature.

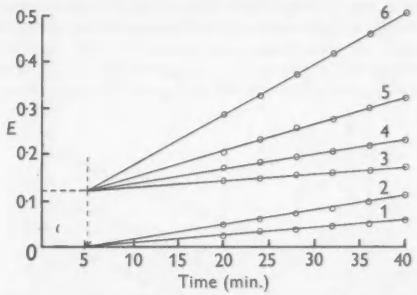

Fig. 2. Extrapolated curves of the colour development of creatinine and creatinine plus creatine. Curve 1: 200 μg. creatinine; curve 2: 400 μg. creatinine; curve 3: 200 μg. creatinine + 10 μg. creatine; curve 4: 400 μg. creatinine + 10 μg. creatine; curve 5: 600 μg. creatinine + 10 μg. creatine; curve 6: 800 μg. creatinine + 10 μg. creatine.

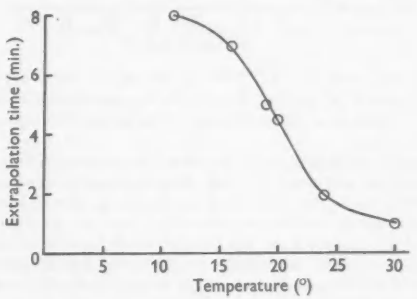

Fig. 3. Variation of the extrapolation time with temperature.

It will be seen from this curve (Fig. 3) that the slope is comparatively steep over the range of temperature normally experienced in the laboratory.

Comparison of the colour densities, as measured at various time intervals after mixing of solutions containing varying amounts of creatine (0, 10, 20, 30 and 40 μg.) in the presence of a constant amount of creatinine (800 μg.), shows (Fig. 4) that the curves so obtained are parallel and that the vertical distance between each succeeding curve corresponds to an E which is equal to that given by the creatine increment alone. Provided then that the temperature is kept constant, and the point at which the time axis is cut by the extrapolated pure creatinine curve is known, it is possible to determine creatine in a mixture of creatine and creatinine. That it is necessary to allow colour development to take

place at constant temperature is apparent from Fig. 3, which shows that a small difference in temperature can produce a marked difference in the extrapolation time and thus lead to a significant error in the estimation.

Urine experiments. Although it was possible to determine creatine in the presence of creatinine in model systems the application of the method to urine analysis, for example, remained unsatisfactory due to the effect of inhibitors other than disulphide or sulphydryl compounds and perhaps to other guanidino compounds which yield coloured products. Although a considerable effort was spent in an attempt to identify the interfering compound(s) we were unable to attribute the interference to a single entity. It was found, however, that substances containing an NH group were, in general, inhibitors of the reaction. A diverse selection of biological and non-biological compounds were tested, and among these piperidine (but not pyridine), glycylglycine, iminodiacetic acid, diketopiperazine, sarcosine, proline and hydroxyproline were effective inhibitors, whereas other common amino acids were comparatively feeble. One of these inhibitors was examined in detail and it will be seen (Fig. 5) that 10 molecular proportions of proline reduce the colour intensity of 13·1 μg. creatine by 45%. It is possible, therefore, that the inhibition in urine is due to the cumulative effect of trivial amounts of a variety of peptides and amino acids as well as to disulphide or sulphydryl compounds.

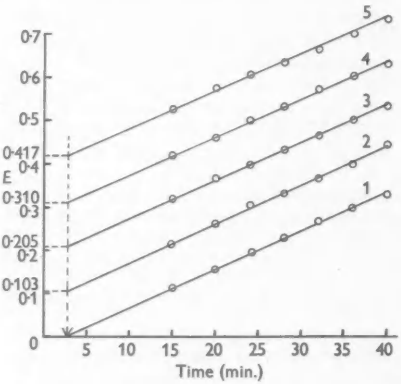

Fig. 4. Extrapolated curves (1–5) given by varying amounts of creatine (0, 10, 20, 30 and 40 μg.) in the presence of a constant amount of creatinine (800 μg.).

We were unable to devise a method which could cope successfully with inhibitors in all specimens of urine. On occasion ion-exchange resins, absorptive or complexing reagents proved useful but no single method was either adequate or convenient for routine analysis. The difficulty was overcome by another approach and we reinvestigated the experimental conditions used by Eggleton *et al.* (1943) with the object of increasing the sensitivity of the method. This was achieved as mentioned above by an increase in the concentration of 1-naphthol in the reaction mixture.

With the resultant increased sensitivity it is important to be certain that the coloured complex arises only from creatine since it is known that other guanidino derivatives may also be excreted. In this connexion Eggleton *et al.* (1943) have reported that under the conditions of this test arginine, glycocyamine and guanidine produce about one-ninth of the colour of an equivalent amount of creatine. We have tested several guanidino compounds, and the results obtained are compared with creatine in Fig. 6.

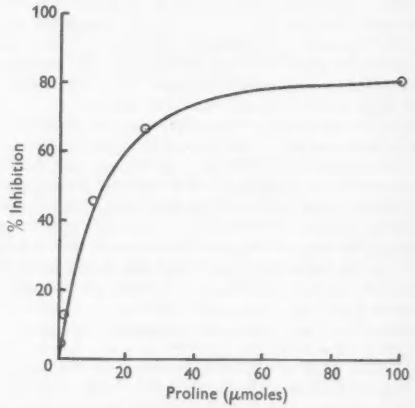

Fig. 5. Inhibition produced by varying molecular proportions (0–100) of proline on the colour given by 1 μmole of creatine. The E was measured 20 min. after the addition of the reagents.

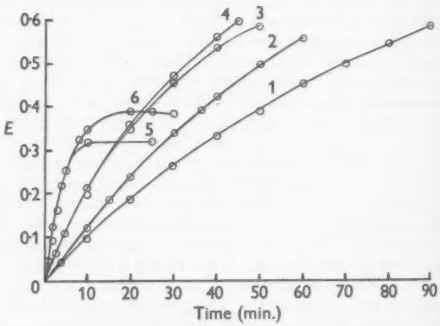

Fig. 6. Comparison of the rates of colour development of substituted guanidines. Curve 1: methylguanidine, 2·5 μmoles; curve 2: glycocyamine, 2·5 μmoles; curve 3: arginine, 2·5 μmoles; curve 4: guanidine, 2·5 μmoles; curve 5: *as.*-dimethylguanidine, 0·25 μmole; curve 6: creatine, 0·25 μmole.

It will be noted (Fig. 6) that of the compounds tested only *as.*-dimethylguanidine ($HN{:}C(NH_2).NMe_2$) reacts in the same way as creatine. On the other hand, the reaction in the case of guanidine, arginine, glycocyamine and methylguanidine shows no sign of reaching completion even in 50 min. At 30 min. these latter compounds require a tenfold concentration to produce the same E as creatine; this finding is in agreement with those of Eggleton *et al.* (1943). Aminoguanidine (5×10^{-3} M) gave no colour.

As discussed in the model experiments it is possible to determine creatine in the presence of creatinine provided that the extrapolation point is known. In urine analysis there are three ways of obtaining the position of this point. (1) Extrapolation to the time axis of the curve obtained by measuring the colour development of a solution of pure creatinine. (2) Intersection of the curves obtained by measuring the colour development of a sample of urine with and without added creatinine (see Fig. 2). (3) Intersection of the curves obtained by measuring the colour development of a sample of urine before and after removal of the creatine present.

The first method has the theoretical disadvantage that the rate of colour development of pure creatinine is not necessarily the same as the rate in urine because of the presence of inhibitors and/or accelerators.

The second method has the obvious advantage of presenting an internally controlled system but will estimate other guanidino compounds if present.

The third method requires for absolute specificity the use of an enzyme system which will attack only creatine. Such a system is found in a pseudomonad described by Nimmo-Smith (1949). This system differs from the bacterial enzyme of Dubos & Miller (1937) in that it has no action on creatinine.

Recommended method

The technique adopted for the enzymic removal of the creatine present in urine was to incubate at 37° for 30 min., with occasional shaking, 0·5 ml. of urine, 0·5 ml. of 0·2 M-phosphate buffer (pH 6·5) and 0·5 ml. of the stock bacterial suspension which contained about 6 mg. dry wt. of organisms/ml. This mixture after incubation was made up to 10 ml., centrifuged and the clear supernatant used for experiments which were arranged in the following fashion:

(1) 2·0 ml. of supernatant (enzyme-treated urine).
(2) 2·0 ml. of supernatant + 5 μg. of creatine.
(3) 2·0 ml. of supernatant + 200 μg. of creatinine.
(4) 0·1 ml. of untreated urine.
(5) 0·1 ml. of untreated urine + 5 μg. of creatine.
(6) 0·1 ml. of untreated urine + 200 μg. of creatinine.

The above arrangement was carried out in duplicate, and to each tube was added 1·0 ml. of *p*-chloromercuribenzoic acid (142 mg./100 ml.) in order to eliminate the inhibitory effect of disulphide or sulphydryl compounds and also to poison small amounts of the enzyme system which remained and which otherwise would rapidly decompose the added creatine. The following reagents were then added at zero time: 2·5 ml. of 1-naphthol solution (640 mg. in 10 ml. of stock alkali) and 1·0 ml. of diacetyl dilution. The mixture was then made up to 10 ml., filtered, and the rate of colour development determined over the period 18–36 min. from zero time. The results of a typical experiment are plotted in Fig. 7.

It will be appreciated that these experiments include both the second and third methods, and moreover that the results are in agreement with each other. Thus the addition of creatinine to either the enzyme-treated urine or the untreated urine (Expts. 1 and 3 and 4 and 6 respectively) leads to results which give an identical extrapolation point. It will also be noted that the same E is obtained for the 5 μg. of creatine which was added to both the treated and the untreated urine (represented by vertical lines marked A and B). The amount of creatine present in the urine is given by C.

Since the curves given by the enzyme-treated urine with and without the addition of creatinine intersect on the time axis it follows that the amount of other guanidino compounds present is insignificant. In other specimens of urine which we have examined the curves intersect above the time axis thus indicating the presence of one or more guanidino compounds, most probably glycocyamine, which is a normal excretory product (cf. Weber, 1935). It will be appreciated, however, that its presence does not interfere with the determination of creatine (provided that the third method of determining the extrapolation point is used) and that the

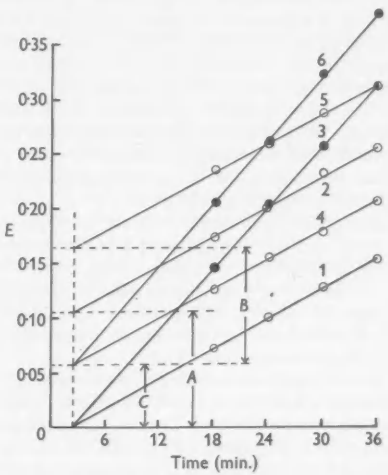

Fig. 7. The estimation of the creatine content of urine. Curve 1: enzyme-treated urine; curve 2: enzyme-treated urine + 5 μg. creatine; curve 3: enzyme-treated urine + 200 μg. creatinine; curve 4: untreated urine; curve 5: untreated urine + 5 μg. creatine; curve 6: untreated urine + 200 μg. creatinine.

method is capable of determining the creatine content in a direct manner. In order to discover the error at different concentrations, analyses were made of normal urine and of urine which had been freed from creatine and subsequently used to make a solution containing 5 μg./ml. In the first experiment the results of duplicate runs on urines of four males and two females were: 19, 23; 22, 21; 25, 26; 24, 28; 8, 5; 24, 21 μg. creatine/ml. At the known lower concentration of 5 μg./ml., 0·25 ml. urine was taken instead of the usual 0·1 ml. and four trial runs gave 4·5, 6·0, 3·3 and 5·3 μg./ml. The error of the method is therefore ±10% at a concentration of 20 μg./ml. and about twice this amount at 5 μg./ml. The lower values were, however, seldom observed; typical values for males were 11, 19, 14, 15, 35, 7, 40, 38, 25, 27, 22, 8 and 12 μg. creatine/ml. urine.

DISCUSSION

There are two essential differences between the method described in this communication and that described by Raaflaub & Abelin (1950). The sensi-

tivity of the method has been increased by rather more than 50% and an accurate assessment has been made of the point at which the extrapolated creatinine curve cuts the time axis. Such an assessment gives the distance along the time axis at which the *E* for creatine must be read and eliminates the error (about 20%) which may arise because of the fact that at ordinary laboratory temperatures the *E*-time curve for pure creatinine does not intersect the time axis at its origin. The point at which the *E* for a solution of pure creatinine cuts the time axis is a function of temperature and the fact that Raaflaub & Abelin (1950) obtained curves passing through the origin could be explained by a slight contamination of creatinine with creatine. The best commercially available specimens of creatinine are not sufficiently pure to be used without further purification; the plots of some commercial samples tested by us have in fact cut the time axis to the left of the origin.

Among those guanidino derivatives which occur naturally and which may reach levels which would lead to interference with the present method only glycocyamine need be considered. If the greatest accuracy is desired, the specific enzymic method must be used since this will lead to a correct assessment of the extrapolation point.

Since an equilibrium exists between creatine and creatinine a solution of the latter will become contaminated with creatine at a rate which is dependent on the temperature and the pH at which it is stored. For this reason it is essential to prepare all solutions of creatinine immediately prior to use. Similar considerations apply to urine specimens which, because of their high creatinine content, will develop increasing amounts of creatine. Thus a specimen of urine, when analysed immediately after voiding, contained 22 μg. of creatine/ml.; after storage at room temperature for 24 hr. it contained 32 μg./ml. In view of this hydrolysis of creatinine it is perhaps pertinent to cast some doubt on the results recorded in the literature for creatine excretion and indeed to query whether or not the creatine which is actually found in small amounts in urine of normal adult males is excreted as such or arises as a result of the hydrolysis of creatinine during the course of the accumulation of urine in the bladder.

SUMMARY

1. The specificity of the Barritt (1936) reaction for the determination of creatine has been investigated.

2. A method is described for the direct determination of creatine in urine.

It is a pleasure to record our thanks to Miss J. D'Alton and to Mr T. Gascoygne for their skilled technical assistance. One of us (A. H. E.) wishes to acknowledge his indebtedness to the Director-General of Health and the Director of the Commonwealth Serum Laboratories for a generous provision of laboratory space pending the transfer of the department to Canberra. We are also very grateful to Dr R. H. Nimmo-Smith for providing us with liberal amounts of the pseudomonad.

REFERENCES

Barritt, M. M. (1936). *J. Path. Bact.* **42**, 441.
Dubos, R. & Miller, B. F. (1937). *J. biol. Chem.* **121**, 429.
Eggleton, P., Elsden, S. R. & Gough, N. (1943). *Biochem. J.* **37**, 526.
Ennor, A. H. & Rosenberg, H. (1952). *Biochem. J.* **51**, 606.
Ennor, A. H. & Stocken, L. A. (1948). *Biochem. J.* **42**, 557.
Ennor, A. H. & Stocken, L. A. (1949). Paper presented to Hobart (Tasmania) meeting of the Australian & New Zealand Association for the Advancement of Science.
Hunter, A. (1928). *Creatine and Creatinine.* London: Longmans Green and Co.
Nimmo-Smith, R. H. (1949). *1st Int. Congr. Biochem. Abstr.* p. 297.
Raaflaub, J. & Abelin, I. (1950). *Biochem. Z.* **321**, 158.
Walpole, G. S. (1911). *J. Physiol.* **42**, 301.
Weber, C. J. (1935). *J. biol. Chem.* **109**, xcvi.
Whitmore, F. C. & Woodward, G. E. (1932). *Org. Synth. Coll.* **1**, 153.

Studies on Sulphatases

4. ARYLSULPHATASE AND β-GLUCURONIDASE CONCENTRATES FROM LIMPETS

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The application of β-glucuronidase concentrates to metabolic studies and biochemical assay is largely dependent on the ease of the hydrolysis of conjugated glucuronides by this agency. Chemical methods of hydrolysis involve boiling the glucuronide with concentrated acid for long periods and degradation of the aglycones under these conditions is not infrequent. It is claimed that greater yields of liberated aglycones are obtained by enzymic than by acid hydrolysis (cf. Samuels & Reich, 1952). Furthermore, the enzymic method is specific for the β-glucuronide linkage whereas acid also liberates hydroxy compounds from the ethereal sulphate conjugates which may also be formed as metabolites. Arylsulphatases are specific for the ester linkage between sulphuric acid and phenolic hydroxyl groups while glucuronidase hydrolyses both aliphatic and aromatic glucuronides. Less attention has been given to the preparation and use of arylsulphatase concentrates than of those of β-glucuronidase. However, commercial fungal preparations possessing weak arylsulphatase activity have proved useful, e.g. Butenandt & Hoffstetter (1939) cite the hydrolysis of oestrone sulphate by Taka diastase as evidence of the presence of an ester sulphate grouping in this compound. Mylase P was employed by Clarke, Akawie, Pógrund & Geissman (1950) (private communication) to demonstrate that the *laevo*-adrenaline conjugate isolated from rabbit urine was not a sulphate ester. Enzymic hydrolysis of pregnant mare's urine with mylase P gave a yield of oestrogens 20% greater than that obtained after acid hydrolysis (Cohen & Bates, 1949, 1950). The method distinguished between oestrogens conjugated with sulphuric acid through phenolic groups and those conjugated with glucuronic acid or with sulphuric acid through aliphatic hydroxyl groups.

The potential value of arylsulphatases in metabolic studies suggested the need for stable, highly active concentrates of the enzyme. Since such concentrates might be used for investigating the metabolism of phenols which are conjugated with both glucuronic and sulphuric acids, it was necessary to prepare arylsulphatase free from β-glucuronidase and this has been the main criterion of purification in the present investigation. A highly active β-glucuronidase preparation contaminated with arylsulphatase has also been obtained. Selective inhibition of the latter enzyme provided a β-glucuronidase concentrate devoid of arylsulphatase activity.

There are four main sources of arylsulphatase, mammalian, molluscan, fungal, and bacterial; of these, molluscan sources yield by far the most active preparations (Table 1). Common molluscs are readily available either commercially or from almost any coast and they invariably possess strong β-glucuronidase activity (Dodgson, Lewis & Spencer, 1953*a*). Of the common molluscs the limpet, *Patella vulgata*, was chosen for study on the grounds of its availability, large size and the ease of access to the visceral hump.

Table 1. *The activity of arylsulphatases from various sources*

(The units of activity are expressed as μg. *p*-hydroxyacetophenone liberated from potassium *p*-acetylphenylsulphate in 1 hr. under the standard conditions (Dogdson *et al.* 1953*a*). The last value quoted represents μg. phenolphthalein liberated by 1 ml. enzyme extract acting on potassium phenolphthalein disulphate under the optimal conditions in 6 hr.)

Type	Source	Activity (units/g.)	Reference
Mammalian	Rat liver Rat kidney	1500–4500 600–900	Dodgson *et al.* (1953*b*)
Molluscan	Visceral hump of *Patella vulgata* and *Littorina littorea*	7000–140 000	Dodgson, Lewis & Spencer, 1952
Fungal	Taka diastase	1300	Dodgson *et al.* (1952)
Bacterial	Extract of *Mycobacterium piscium*	59	Whitehead, Morrison & Young, 1952

EXPERIMENTAL AND RESULTS

Arylsulphatase concentrates have already been prepared by Soda & Egami (1941) from the digestive glands of *Charonia lampas* (*Tritonium nodiferum*), a large tropical marine mollusc, but it is not stated whether separation from β-glucuronidase was achieved. These workers autolysed the macerated material for several days but this was considered inadvisable in the present experiments since buffered suspensions (pH 5·5) of ground *Patella* viscera rapidly lose arylsulphatase activity at 37·5° (Dodgson *et al.* 1953*a*).

In order to obtain a stable final concentrate in powder form all fractions have been acetone-dried. Although this results in some loss of the enzyme it has the advantage of convenience of procedure and ease of repetition with simple equipment.

Preparation of acetone-dried limpet viscera. Inactivation of the enzyme during concentration was kept as low as possible by keeping the acetone and all solutions at 0°. Acetone precipitations of the soluble enzyme were carried out by the slow addition of acetone (60%, v/v, final concentration) to the rapidly stirred solution. The mixtures were then left at 0° for 15 min. before the precipitates were collected.

Limpets from Sully, Glamorgan, were starved for 5–6 days in aerated sea water. This procedure is advisable in order to remove the main bulk of the food material from the viscera. After being washed quickly in water, 100 limpets (906 g.) were removed from their shells and the visceral humps dissected out. The pooled viscera (122 g.) were macerated in 800 ml. of acetone using a chilled Townson and Mercer macerator. The macerate was quickly filtered by suction, the debris transferred to a further 600 ml. of acetone and the maceration and filtration repeated. The debris was washed three times with 50 ml. of acetone, sucked free from liquid and dried overnight *in vacuo*. The light-grey powder (powder *A*, 22·5 g.) was assayed for arylsulphatase and β-glucuronidase activity using potassium *p*-acetylphenylsulphate and *p*-chlorophenylglucuronide respectively as substrates (Dogdson *et al.* 1953*a*). It was advantageous to suspend the powder in buffer by means of a glass homogenizer with a smooth-ended pestle. The activities were: arylsulphatase 375 000 units/g., β-glucuronidase 200 000 units/g. (A unit of activity is that which liberates 1 μg. of the phenol from the substrate in 1 hr. under optimal conditions.)

The values quoted were obtained from limpets collected in June and it should be emphasized that the enzyme activities of acetone-dried preparations vary, depending on the month of collection, the length of starvation and on other unknown factors. The powder was stable for several months when kept in an air-tight bottle at 0°.

Purification of the acetone-dried viscera. Centrifugal fractionation of aqueous suspensions of powder *A* showed that about 95% of the arylsulphatase was associated with the supernatant whilst the bulky residue was almost devoid of activity. Powder *A* (60 g.) was suspended in 150 ml. of water using a pestle and mortar. (Shaking or the use of macerators and glass homogenizers led to troublesome frothing.) The suspension was centrifuged and the protein of the clear supernatant precipitated with acetone. The main bulk of this supernatant was decanted, the remainder filtered and the residue, while still wet, was washed well with acetone and the latter finally removed *in vacuo*. (The filter-cake must not be allowed to crack until it has been thoroughly washed and should be water-free before the vacuum treatment in order to avoid the formation of gums.) The buff-coloured powder obtained (powder *B*, 8·67 g.) had an arylsulphatase activity of 1 810 000 units/g. This represents a concentration of 4·82 times with a retention of 70% of the initial activity of powder *A*. Glucuronidase activity was 690 000 units/g. (concentration 3·45 times; 50% of initial activity).

Removal of β-glucuronidase. Soda & Egami (1941) used trypaflavine (or euflavine; 2:8-diaminomethylacridinium chloride) for the purification of the sulphatases of *Charonia lampas* but they did not study its action on glucuronidase. Similar treatment of powder *B* with this flavine concentrated both the arylsulphatase and the glucuronidase (Table 2). Further investigation of the action of flavines (substituted acridines) was undertaken in the hope that a method of removal of β-glucuronidase would be found. Powder *B* (1 g.) was ground in a mortar with 35 ml. of ice-cold water and 0·65 g. of flavine. The precipitated material and undissolved flavine were spun down and the supernatant solution precipitated with acetone. This precipitate was collected by centrifuging and suspended in 20 ml. of water. The solution was centrifuged to remove undissolved material, precipitated with acetone and dried *in vacuo*. Unless otherwise stated, the pH of the original mixture was controlled by the excess of flavine present.

The results obtained with the different flavines are summarized in Table 2. Treatment with proflavine sulphate (2:8-diaminoacridine disulphate) at pH 2·9 removed almost all the glucuronidase and concentrated the arylsulphatase.

Table 2. *The enzyme activity of powder* B *after treatment with flavines*

(Powder *B* (1 g.) was treated with flavine (0·65 g.) or the pH adjusted as described in text.)

			Arylsulphatase activity		Glucuronidase activity	
Flavine	pH of mixture	Yield (g.)	(Units/g.)	(% of initial activity)	(Units/g.)	(% of initial activity)
Acriflavine	1·8	0·170	430 000	4·0	0	0
None	1·8*	0·178	3 760 000	37·0	0	0
Proflavine sulphate	2·9	0·128	5 660 000	39·6	78 000	1·4
Proflavine hemisulphate	2·9*	0·201	3 880 000	43·0	28 500	0·8
Euflavine	2·9*	0·212	4 010 000	47·0	40 700	1·3
None	2·9*	0·139	4 580 000	35·2	65 000	1·3
Proflavine hemisulphate	6·5	0·250	3 330 000	42·2	1 984 000	48·7
Euflavine	6·5	0·200	2 900 000	32·1	1 300 000	26·0
None	6·5*	0·292	2 570 000	41·6	688 000	37·9

* The pH of the mixture was adjusted to this pH.

Proflavine hemisulphate and euflavine (pH 6·5) concentrated both enzymes whilst a commercial specimen of acriflavine (2:8-diaminoacridine hydrochloride containing 2:8-diaminomethylacridinium chloride hydrochloride) at pH 1·8 removed all the glucuronidase and almost all the arylsulphatase. These results were mainly due to pH effects since the results obtained with any flavine at its natural pH could be achieved with other flavines if the pH of the flavine-powder *B* aqueous mixture was suitably adjusted. Furthermore, similar treatment of aqueous suspensions of powder *B*, involving pH adjustment with dilute H_2SO_4 in the absence of flavine, resulted in powders having almost identical properties with those prepared using the respective flavines (Table 2). Only in the case of acriflavine was any difference in enzyme activity shown between powders prepared in the presence and absence of flavines.

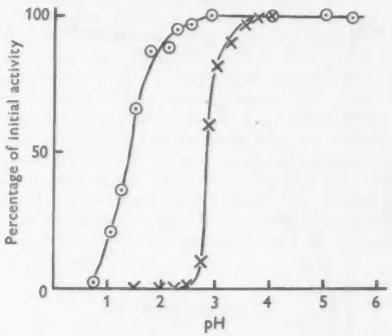

Fig. 1. The effect of pH on the arylsulphatase and β-glucuronidase of an aqueous solution of powder *B*. Portions of the solution were adjusted to various pH's, centrifuged and the precipitates and supernatant solutions assayed. At all pH's the activity of the precipitate was negligible. The β-glucuronidase (×—×) and arylsulphatase (⊙—⊙) activities of the supernatant solutions are shown.

The effect of pH on the enzymes of powder *B* was further investigated. Aqueous suspensions of powder *B* were adjusted to various pH's with dilute HCl, centrifuged, the precipitates washed with water adjusted to the same pH, centrifuged again and the residue and combined supernatants assayed. All solutions were kept ice-cold during manipulation before incubation with substrate. Fig. 1 shows that the glucuronidase and arylsulphatase activities of the supernatant solution declined sharply at low pH. There was no corresponding increase in the activities of the precipitate. It must be assumed that irreversible inactivation of the enzymes takes place at acid pH even at 0°. Combination of the supernatant and residue before assay did not increase the recovery. The inactivation of both enzymes was not progressive with time since there was no further decline in activity after the first sample had been taken (about 1 min. after pH adjustment). It is important, therefore, in adjusting limpet viscera extracts to acid pH, that the required pH is not overshot on the acid side. The β-glucuronidase of powder *B* is inactivated at higher pH than the arylsulphatase and at pH 2·25 it is entirely removed whilst about 95% of the arylsulphatase remains.

Purification of powder *B* (1 g.), using the method employed with the flavines but adjusting to pH 2·25 and omitting the flavine yielded 0·188 g. of a stable glucuronidase-free powder (powder *C*) having an arylsulphatase activity of 3650000 units/g. (37·9% of the initial activity).

Simplified method. For routine purposes the preparation of glucuronidase-free arylsulphatase concentrates from acetone-dried limpet viscera was simplified. Powder *A* (25 g.) was ground in a mortar with water (200 ml.). The suspension was adjusted to pH 2·25 and the undissolved and precipitated material removed by centrifuging. Acetone was added to the supernatant and the precipitate formed collected by centrifugation. This was suspended in water (100 ml.), undissolved material was spun down, and the arylsulphatase, free from glucuronidase, was precipitated from the solution by acetone. The precipitate was collected, washed with acetone on the vacuum filter and finally dried *in vacuo*. Yield, 1·15 g.; arylsulphatase activity, 3680000 units/g.; concentration, 9·7-fold with a retention of 46·4% of the initial activity. The white powder (powder *D*) dissolved easily in water or 0·5 M-acetate buffer, pH 5·5.

Characteristics of the purified preparation. The optimal conditions for the arylsulphatase of powder *D* were substantially the same as those determined in crude suspensions of limpet viscera (Dodgson *et al.* 1953*a*). The optimum pH (5·5) was unchanged but the optimum substrate concentration was 0·055 M instead of 0·065 M. Incubation of the purified preparation without substrate at 37·5° for 1 hr. did not inactivate the enzyme. The graph of enzyme concentration against activity was a straight line but the activity-time curve showed that the initial hydrolysis rate declined during the first 45 min. to a steady value which was maintained for at least 2 hr.

The activity of arylsulphatase concentrates in urine and the effect of various ions

A possible use of arylsulphatase concentrates is for the hydrolysis of urinary ethereal sulphates. The effect of urine on the activity of purified glucuronidase-free arylsulphatase preparations was therefore investigated. The enzyme was inhibited strongly. In a typical experiment rabbit urine caused 91·5% inhibition and human urine 87·8%. In another experiment a mixture of rabbit urine (10 ml.) at pH 5·5 and 0·5 M-acetate buffer (10 ml.) at the same pH containing 70·6 mg. of potassium *p*-acetylphenylsulphate was incubated with powder having an activity of 20000 units. After 1 hr. there was 8·5% hydrolysis and after 16 hr. only 20%. In the absence of urine, hydrolysis was complete in 11·5 hr. The cause of the inhibition of arylsulphatase was traced by testing the effect of several common urinary constituents on the enzyme (Table 3). Urea appeared to have no influence on arylsulphatase action and the various chlorides had a slight activating effect. The inhibition by urine could be attributed, however, to the presence of sulphate and

phosphate ions, both of which were strong arylsulphatase inhibitors. The inhibition by phosphate was not due to a shift in pH maximum of the enzyme from that of 5·5 in acetate buffer since the purified limpet preparations were entirely inactive in 0·2 M-phosphate buffer at pH's 5–9. Both Taka diastase and rat-liver arylsulphatase have different pH optima in phosphate and in acetate buffers (Dodgson, Spencer & Thomas, 1953*b*).

Table 3. *The effect of various compounds on the arylsulphatase activity of limpet viscera concentrates*

(Substrate, 0·055 M-potassium *p*-acetylphenylsulphate in 0·5 M-acetate buffer, pH 5·5, incubated 1 hr. at 37·5°.)

Compound	Molarity	Arylsulphatase activity (%)
None	—	100
Urea	0·02	98·6
NaCl	0·01	109·0
	0·02	110·3
KCl	0·02	111·3
K_2SO_4	0·01	56·3
	0·02	56·2
KH_2PO_4	0·02	0
Phenylhydrazine hydrochloride	0·00005	103·3
	0·0005	124·0
$MgCl_2$	0·02	109·3
$BaCl_2$	0·1	107·5
Magnesium acetate	0·02	97·8

Table 4. *The activity of the arylsulphatase of limpet viscera concentrates in the presence of treated urines*

(Enzyme in 0·6 ml. M-acetate buffer was incubated with substrate dissolved in 0·6 ml. of the solutions listed.)

Solution	Activity (%)
Water	100·0
0·2 M-$BaCl_2$	110·0
Urine	20·3
0·2 M-$BaCl_2$ in urine	20·1
Urine precipitated at pH 5·5 with 0·2 M-$BaCl_2$ and the precipitate removed by centrifugation	18·9
Urine precipitated at pH 11·5 with 0·2 M-$BaCl_2$ and the precipitate removed by centrifugation	106·0

Removal of inhibiting substances from urine. The inhibitory effect of urine is sufficient to reduce the arylsulphatase activity of limpet concentrates to an inefficient level, and it is necessary to remove the sulphate and phosphate ions before the use of the concentrates in urine is practicable. Both ions may be removed by precipitation with barium chloride at pH 11·5 and urine treated in this manner is no longer inhibitory (Table 4). Precipitation of the sulphate ions only by barium chloride at pH 5·5 does not reduce the inhibitory effect of urine even after removal of the precipitated barium sulphate by centrifugation. Phosphate ions must, therefore, be regarded as the main cause of the inhibition. The sulphate- and phosphate-free urine has a slight activating effect which may be attributed to the increased concentration of chloride ions added as barium chloride.

The following experiments show that arylsulphates present in urine are unaffected by the barium chloride treatment and that subsequently they can be hydrolysed to completion by the enzyme concentrates.

Rabbit urine (25 ml.) containing potassium *p*-acetylphenylsulphate (100 mg.) was adjusted to pH 11·5 with concentrated KOH and solid $BaCl_2$ (0·52 g.) added. The precipitated barium salts were removed by centrifugation and

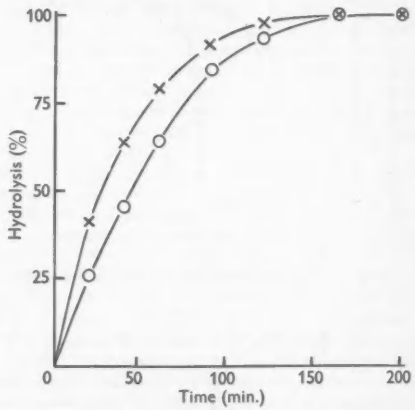

Fig. 2. The hydrolysis of potassium *p*-nitrophenylsulphate (×—×) dissolved in treated human urine and potassium *p*-acetylphenylsulphate (○—○) dissolved in treated rabbit urine by purified limpet arylsulphatase powder *D* at 37·5°. The sulphate and phosphate ions of both urines were removed as barium salts at pH 11·5 and the urines were then adjusted to pH 5·5.

15 ml. of the clear supernatant were adjusted to pH 5·5 with conc. HCl and made up to 25 ml. with 0·5 M-acetate buffer at the same pH. To this solution was added purified limpet arylsulphatase powder (73 600 units) and the mixture incubated at 37·5°. Portions were removed at intervals, enzyme action was stopped with 0·2 N-NaOH and the liberated *p*-hydroxyacetophenone determined. A similar experiment was carried out with potassium *p*-nitrophenylsulphate (100 mg.) dissolved in human urine (25 ml.). Liberated *p*-nitrophenol was measured spectrophotometrically at 400 mμ. (Dodgson & Spencer, 1953). The results are shown graphically in Fig. 2. The sulphates of *p*-nitrophenol and *p*-hydroxyacetophenone were hydrolysed completely in 148 and 160 min. respectively.

It may be concluded that the use of limpet arylsulphatase concentrates for the hydrolysis of arylsulphates in urine is efficient provided that inhibiting ions are previously removed.

Suppression of arylsulphatase activity of concentrates containing β-glucuronidase

The complete inhibition of limpet arylsulphatase by 0·1 M-phosphate and the failure of this concentration of phosphate to affect limpet glucuronidase activity suggested that powder *B* could be used as a β-glucuronidase concentrate, free from arylsulphatase activity, without actually removing the arylsulphatase.

Sodium dihydrogen phosphate was added to human urine to give a 0·2 M solution which was adjusted with HCl to pH 4·0, the optimum pH of limpet viscera glucuronidase, and then diluted with an equal volume of 0·5 M-acetate buffer at the same pH. To one portion of this solution potassium *p*-acetylphenylsulphate (1 mg./ml.) was added and to another portion *p*-chlorophenylglucuronic acid (1 mg./ml.). Powder *B* (1 mg./ml.) was added to each portion which was then incubated at 37·5°, and 1·2 ml. samples removed at intervals and deproteinized with 4·8 ml. ethanol. After centrifugation, 5 ml. of the clear supernatants were each added to 1 ml. N-NaOH and the liberated phenols in the resultant mixtures determined. Complete hydrolysis of *p*-chlorophenylglucuronic acid occurred after 2 hr. No *p*-hydroxyacetophenone was liberated from its sulphate even after 24 hr. Powder *B* can therefore be used in urine as an arylsulphatase-free β-glucuronidase preparation provided that phosphate ions (0·1 M) are present.

DISCUSSION

The high arylsulphatase and β-glucuronidase contents of limpet viscera permit the preparation of concentrates of these enzymes which compare favourably in activity and ease of preparation with those derived from other sources. Powder *B* can be used as a mixture of the two enzymes or, in the presence of 0·1 M-phosphate, as a β-glucuronidase concentrate free from arylsulphatase activity. Powders *C* and *D* contain glucuronidase-free arylsulphatase. These powders can be used to release phenols from their glucuronide and sulphate conjugates either simultaneously or separately and may, therefore, prove useful for the hydrolysis of urinary steroid conjugates as a preliminary to steroid analysis. In urine the use of the concentrates is limited if precipitation of phosphate and sulphate ions by barium hydroxide results in the removal of the arylsulphate under study. However, none of the common arylsulphates encountered by the authors is precipitated under these conditions.

The adoption of the term 'arylsulphatase' for the sulphatase of limpet viscera is based on its ability to hydrolyse certain arylsulphates. The term implies that the hydrolytic activity of the enzyme is confined to the ester linkage between sulphuric acid and hydroxyl groups of phenolic character. This assumption of specificity has not yet been rigorously tested but is reasonable if the specificities of the arylsulphatases of mammals, fungi and, especially, tropical marine molluscs are borne in mind. It should be noted that the absence of β-glucuronidase from the arylsulphatase concentrates is the only step towards enzymic purity here claimed. Other enzymes are undoubtedly present and, in certain circumstances, these may limit the usefulness of the concentrates.

Limpet arylsulphatase is strongly inhibited by urine whilst the enzyme of Taka diastase and other fungal preparations is only weakly affected (Noguchi, 1923; Dzialoszynski, 1947). This inhibition is due to phosphate and sulphate ions, since at 0·02 M concentration these inhibit the limpet enzyme 100 and 43·7 % respectively, and the arylsulphatase of fungal origin only 4–19 and 0 % respectively (Robinson, Smith, Spencer & Williams, 1952). Dzialoszynski attributes the inhibitory effect of urine on the sulphatase of Clarase to urea which inhibited the enzyme in pure buffer solution. Urea (0·02 M) had negligible action on limpet arylsulphatase. The effects of other ions have revealed further differences between the arylsulphatases from various sources. Chloride ions activate the limpet enzyme (Table 3) and rat-liver arylsulphatase (Dodgson *et al.* 1953*b*) but have no effect on the arylsulphatase of Taka diastase (Robinson *et al.* 1952). Seligman, Chauncey & Nachlas (1951) state that magnesium chloride activates rat-liver arylsulphatase by 50 %, but this action is much less with the limpet enzyme. The activation of limpet concentrates by magnesium chloride can probably be attributed to chloride ions since magnesium acetate is almost without effect. Dzialoszynski (1950) found that 0·00001 M-phenylhydrazine completely inhibited the arylsulphatase of Clarase, whereas phenylhydrazine hydrochloride activated limpet viscera concentrates.

The effect of inorganic ions upon arylsulphatases from various sources combined with the different kinetic behaviour of the enzymes towards the same substrate (Dodgson *et al.* 1953*a*) suggests that there are basic differences between arylsulphatases. It must not be assumed, therefore, as it has been assumed in the past, that conclusions based on the study of an arylsulphatase from one source are directly applicable to the enzymes from other sources.

SUMMARY

1. Acetone-dried preparations of limpet viscera were fractionated to give stable powders containing both arylsulphatase and β-glucuronidase.

2. Irreversible inactivation of the β-glucuronidase of these powders at pH 2·25 yielded a stable arylsulphatase preparation free from β-glucuronidase.

3. The inhibition of limpet arylsulphatase by

urine can be removed by precipitation of the urine with barium chloride and may be attributed to phosphate and sulphate ions.

4. Total inhibition of limpet arylsulphatase by 0·2 M-phosphate allows the use of the crude viscera concentrates as β-glucuronidase preparations free from arylsulphatase.

5. The dissimilarity in the characteristics of arylsulphatases from various sources is commented on.

REFERENCES

Butenandt, A. & Hoffstetter, H. (1939). *Hoppe-Seyl. Z.* **259**, 222.

Clarke, W. G., Akawie, R. J., Pógrund, R. S. & Geissman, T. A. (1950). *18th Int. Congr. Physiol. Abstr.* p. 159.

Cohen, H. & Bates, R. W. (1949). *Endocrinology*, **44**, 317.

Cohen, H. & Bates, R. W. (1950). *Endocrinology*, **45**, 86.

Dodgson, K. S., Lewis, J. I. M. & Spencer, B. (1952). *Biochem. J.* **51**, xlii.

Dodgson, K. S., Lewis, J. I. M. & Spencer, B. (1953*a*). *Biochem. J.* **55**, 254.

Dodgson, K. S. & Spencer, B. (1953). *Biochem. J.* **53**, 444.

Dodgson, K. S., Spencer, B. & Thomas, J. (1953*b*). *Biochem. J.* **53**, 452.

Dzialoszynski, L. M. (1947). *Nature, Lond.*, **160**, 464.

Dzialoszynski, L. M. (1950). *Nature, Lond.*, **166**, 157.

Noguchi, J. (1923). *Biochem. Z.* **143**, 186.

Robinson, D., Smith, J. N., Spencer, B. & Williams, R. T. (1952). *Biochem. J.* **51**, 202.

Samuels, L. T. & Reich, H. (1952). *Annu. Rev. Biochem.* **21**, 129.

Seligman, A. M., Chauncey, H. H. & Nachlas, M. M. (1951). *Stain Tech.* **26**, 19.

Soda, T. & Egami, F. (1941). *J. chem. Soc., Japan*, **62**, 256.

Whitehead, J. E. M., Morrison, A. R. & Young, L. (1952). *Biochem. J.* **51**, 585.

The Oxidation of Glycerol by Mycobacteria

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Nocard & Roux (1887) discovered that the growth of *Mycobacterium tuberculosis* var. *hominis* was stimulated by addition of glycerol to culture media, but Hammerschlag (1891) was the first to demonstrate utilization of glycerol. Several investigators have since shown that glycerol is a favourable source of carbon for the cultivation of many species of mycobacteria. Baudran (1910) drew attention to the simultaneous disappearance of glycerol and phosphoric acid from culture media, and suggested that glycerophosphoric acid might be formed. Merrill (1930, 1931*a*, *b*) studied the fate of glycerol in broth cultures and in synthetic media, and concluded that glycerol was completely oxidized without the accumulation of breakdown products, a view which was shared by Dingle & Weinzirl (1932), Wedum (1936) and Friedemann & Seibert (1939).

The investigation of washed cell suspensions has furnished additional information (see Edson, 1951). Nakamura (1938) reported that glycerol increased the oxygen consumption of *Myco. tuberculosis* var. *hominis*; but no direct relationship between respiration and the growth-promoting ability of glycerol could be found by Bloch, Matter & Suter (1947). Although it is clear that many acid-fast organisms oxidize glycerol, there has been little advance in knowledge of the intermediate metabolism. This paper shows that the use of washed-suspension techniques and cell-free extracts has provided means of elucidating the mechanism of glycerol degradation.

METHODS

Organisms. The organisms used were *Myco. stercoris* (no. 3820), *Myco. smegmatis* (no. 523) and *Myco. butyricum* (no. 337). These strains were obtained from the National Collection of Type Cultures, Lister Institute, London. Stock cultures were maintained on glycerinated Dorset's egg medium, and cultivated on meat infusion broth containing 1% (w/v) peptonum B.P.C. (British Drug Houses) and 5% (v/v) glycerol. On the fifth or sixth day of incubation at 38°, the bacteria were washed and suspended in distilled water by methods described earlier (Edson & Hunter, 1943).

Bacterial extracts. Extracts were prepared by disrupting the organisms either in the wet crushing mill of Booth & Green (1938) or in the Mickle disintegrator (H. Mickle, Hampton, Middlesex). In the Mickle apparatus 4 g. of washed cells, 4 g. of no. 12 ballotini glass spheres (0·18–0·25 mm.) and 4 ml. of phosphate buffer (0·1 M, pH 7·4) were mixed, precooled in each container and shaken at maximum amplitude for 30 min. in a cold room (2–3°). The suspension of disintegrated organisms and glass beads was diluted with 4 ml. of buffer per tube, the extracts clarified by centrifugation and the supernatant dialysed for 24–48 hr. against distilled water at 2–3°.

Manometric measurements. Oxygen consumption was measured by the Warburg technique at 38°, and the method of Warburg & Yabusoe (1924) was used for the determination of the respiratory quotient (R.Q.). Substrates were added from the side bulb after equilibration, and inhibitors were in contact with bacteria from the start of an experiment. The CO_2 absorbent consisted of appropriate mixtures of KOH and KCN (Krebs, 1935) when HCN was used as inhibitor. Acid substrates were used as sodium salts.

Materials. Adenosine triphosphate (ATP) was a purified sample supplied by Boots Pure Drug Co. Ltd. The barium salt was about 94% pure as judged by 7-min. P, 7-min. P/total P and P/N values. A typical analysis of the solution (sodium salt) used in experiments gave: inorganic P, <0·05 mg.; acid-labile 7-min. P, 0·71 mg.; total P, 1·10 mg.

Diphosphopyridine nucleotide was obtained from Schwartz Laboratories Inc., New York. Its purity (50%) was assayed by the method of LePage (1947), using the value $E^{1\%}_{1\,cm.} = 8500$ at 340 mμ.

Analytical methods

Estimation of glycerol. At the end of an experiment with intact cells, the Warburg flasks were cooled in ice and the contents quantitatively transferred to a graduated test tube by means of a Pasteur pipette. Deproteinizing reagent (0·4 ml. of 7% (w/v) metaphosphoric acid solution) was added, the volume adjusted to 10 ml. with distilled water, the tubes centrifuged and 2·0 ml. portions of supernatant used for analysis by the periodate method of Voris, Ellis & Maynard (1940).

Determination of phosphate and phosphate esters. After experiments with clarified and dialysed extracts, the flasks were cooled in ice, 1 ml. of 30% (w/v) trichloroacetic acid was added and the volume made up to 10 ml. with distilled water. After centrifugation 2 ml. portions of the supernatant were used for phosphorus determinations. Triose phosphate was determined as alkali-labile P (Lohmann & Meyerhof, 1934) and ATP as readily hydrolysable P (7 min., 1·0 N-HCl, 100°). For total-P determinations the wet incineration was carried out by heating 1 ml. of P-containing solution with 0·5 ml. of H_2SO_4 (sp.gr. 1·84) and 0·5 ml. of HNO_3 (sp.gr. 1·42) in a micro-Kjeldahl flask. Additional HNO_3 was added if required. All phosphate determinations (inorganic P, hydrolysable P and total P) were performed by the method of Fiske & Subbarow (1925). Phosphoenolpyruvic acid and pyruvic acid were measured by the methods of Lohmann & Meyerhof (1934) and Friedemann & Haugen (1943) respectively.

RESULTS

Respiration

The oxygen consumption of *Myco. smegmatis*, *Myco. stercoris* and *Myco. butyricum* in the presence and absence of relevant substrates is shown in Table 1. The endogenous respiration of each organism is substantial and fairly constant. For a series of different cultures the mean Q_{O_2} ($Q_{O_2} = \mu l.$ O_2 consumed/mg. dry wt./hr.) values (with S.D.) in the absence of added substrate were 13·4 ± 0·9 (twenty-two cultures) for *Myco. smegmatis*, 10·1 ± 0·6 (nine cultures) for *Myco. stercoris* and 13·7 ± 0·8 (twenty-three cultures) for *Myco. butyricum*. In the presence of glycerol the oxygen consumption was doubled. Working with a strain of *Myco. tuberculosis* var. *hominis* virulent for guinea pigs, Bloch (1944) and Bloch *et al.* (1947) reported that the augmentation of oxygen uptake due to glycerol was little affected by the pH of the medium. *Myco. smegmatis* and *Myco. butyricum* also behave in the same way. When the medium was buffered at pH 7·8, 6·8 or 6·2, there was little effect on the extra oxygen uptake due to glycerol, although the endogenous respiration was diminished at the lower pH.

Table 1. *Oxygen consumption of mycobacteria in the presence of various substrates*

(Washed bacteria, 12·0–13·0 mg. dry wt./cup suspended in 3·3 ml. of 0·03 M-phosphate buffer, pH 7·4, at 38°; gas, air. $Q_{O_2} = \mu l.$ O_2 uptake/mg. dry wt. bacteria/hr.)

Substrate (0·02 M unless stated otherwise)	Q_{O_2}		
	Myco. smegmatis	*Myco. stercoris*	*Myco. butyricum*
Nil	15·0	11·9	13·4
Glycerol	32·0	20·5	25·3
DL-Glyceraldehyde	54·8	42·8	21·9
Dihydroxyacetone	41·1	22·8	18·0
Glycerate	32·7	24·3	17·4
α-Glycerophosphate (0·01 M)	18·5	13·8	13·6
D-3-Phosphoglycerate (0·01 M)	15·0	12·7	12·9
Pyruvate	33·2	23·6	25·0
Acetate	38·7	23·1	33·8
Glucose	20·0	15·9	16·6

Oxygen consumption is considerably increased by the presence of DL-glyceraldehyde, dihydroxyacetone, pyruvate and acetate. Although the suspensions do not oxidize α-glycerophosphate or phosphoglycerate significantly, it will be shown later that cell extracts are able to bring about the degradation of these substances. Bacterial suspensions which had been heated in a boiling-water bath for 10 min. did not take up oxygen either in the presence or absence of glyceraldehyde or dihydroxyacetone. Hence the high oxygen consumption in the presence of these relatively unstable substrates is not due to spontaneous oxidation.

Disappearance of glycerol in relation to oxygen uptake. The relationship between oxygen consumption and the disappearance of glycerol was investigated by two methods: (1) The rate of oxygen uptake in washed suspensions was measured in the presence of small amounts of glycerol and compared with the endogenous respiration. The resulting curves for *Myco. smegmatis* are given in Fig. 1. It will be seen that the graphs are rectilinear up to the point where glycerol disappears completely from the medium, and thereafter become parallel with the curve for the control suspension to which sub-

strate was not added. The excess oxygen uptake at the point where the rate of gas absorption became equal to the endogenous rate was used in calculating the ratio oxygen consumed/glycerol disappearing. In Fig. 1 the molar ratios of oxygen consumed/glycerol disappearing are 1·6, 1·5 and 1·4 for curves *A*, *B* and *C* respectively. *Myco. butyricum* gave

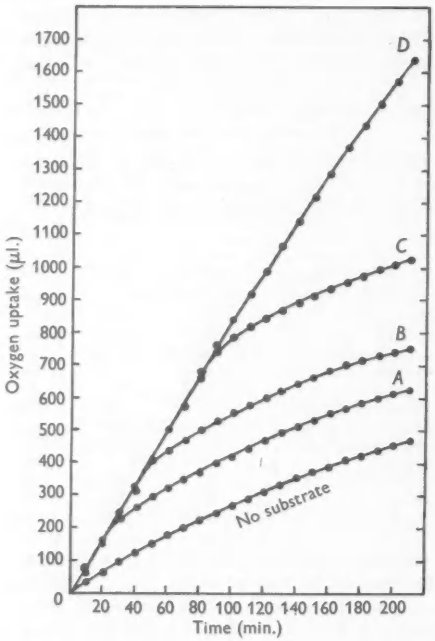


Fig. 1. Oxygen consumption of washed suspension of *Myco. smegmatis* (9·0 mg. dry wt. bacteria/3·4 ml. 0·03 M-phosphate buffer, pH 7·4, at 38°; gas, air). Initial quantity of glycerol: *A*, 4·0 µmoles; *B*, 8·0 µmoles; *C*, 16·0 µmoles; *D*, 40·0 µmoles.

similar curves with the same amounts of glycerol, the molar ratios being 1·0, 1·1 and 1·2 respectively. (2) Residual glycerol was estimated after suspensions of the organisms had respired for a fixed time in a buffered medium containing excess of glycerol (Table 2).

The results of both methods agree in demonstrating that glycerol is incompletely oxidized, the molar ratio of oxygen uptake/glycerol consumed being less than half the theoretical value for complete oxidation in one case (*Myco. smegmatis*) and less than a third in the other (*Myco. butyricum*). The assimilation of glycerol resembles that of glucose, lactate (Edson & Hunter, 1947), pyruvate and fatty acids (Lindsay, O'Donnell & Edson, 1950), all of which are incompletely oxidized by washed suspensions of mycobacteria.

Oxygen consumption and carbon dioxide production. Table 3 records the results obtained when the oxygen consumption and carbon dioxide production of washed suspensions were measured in the presence and absence of glycerol. The 'extra' carbon dioxide/oxygen ratios, which are lower than the theoretical R.Q. for complete combustion of glycerol, suggest that oxidative assimilation may lead to the formation of carbohydrate and/or fatty acids.

Influence of inhibitors on respiration. Table 4 shows the effect of various reagents on the oxidation of glycerol by *Myco. smegmatis*, *Myco. stercoris* and *Myco. butyricum*. Typical data illustrating the influence of fluoride and fluoroacetate on the oxidation of glycerol, pyruvate and acetate are recorded in Table 5. The inhibition of respiration referred to below is calculated on the basis of oxygen uptake in excess of that due to endogenous respiration.

It will be seen that the endogenous respiration is strongly inhibited by cyanide and arsenite, the profound effect of cyanide on the oxidation of glycerol indicating that the oxidation may be linked to the cytochrome system. Arsenite blocks the oxidation of pyruvate almost completely. It also inhibits the oxidation of glycerol by 72 to 80% in *Myco. smegmatis* and *Myco. stercoris*, and to a lesser degree (49 to 62%) in *Myco. butyricum*. Glyceraldehyde oxidation is likewise sensitive to arsenite, the effect being more pronounced in *Myco. butyricum*. Iodoacetate inhibits glycerol oxidation by 63–80%. Malonate has no significant effect. Fluoride, which has no inhibitory action at pH 7·4, strongly inhibits the oxidation of glycerol at pH 6·0 in *Myco. smegmatis*, but does not affect this oxidation in *Myco. butyricum*. Fluoride also affects the metabolism of pyruvate and acetate with results which depend on pH and on the species of organism (cf. the observation of Fitzgerald & Bernheim (1948) on the tubercle bacillus). In *Myco. smegmatis* fluoride causes partial inhibition of pyruvate (24%) and acetate (62%) oxidation at pH 6·0 but not at pH 7·4; whereas in *Myco. butyricum* at pH 6·0 there is partial inhibition of acetate oxidation (21%) and a slight stimulation of pyruvate oxidation. These differences in the sensitivity of species to selective metabolic inhibitors are well illustrated in the case of fluoroacetate, the action of which is not greatly affected by pH.

The interpretation of these facts is difficult, but taken together they suggest that glycerol may be phosphorylated and broken down to pyruvate via the relevant portion of the Embden-Meyerhof scheme.

Table 2. *Oxidation of glycerol by washed suspensions of mycobacteria*

(Bacteria suspended in 3·4 ml. 0·03 M-phosphate buffer, pH 7·4, at 38°. Gas, air.)

Time (min.)	Initial glycerol (μmole)	Oxygen uptake (μmole)	'Extra' oxygen consumed (μmole)	Glycerol disappearing (μmole)	μmole 'extra' oxygen used / μmole glycerol disappearing
		Myco. smegmatis (7·0 mg. dry wt.)			
60	0·00	8·40			
	79·43	20·76	12·36	8·98	1·4
120	0·00	15·90			
	79·18	41·57	25·67	16·87	1·5
180	0·00	22·77			
	78·79	63·89	41·12	27·50	1·5
		Myco. butyricum (15·7 mg. dry wt.)			
60	0·00	7·72			
	78·24	15·00	7·28	7·22	1·0
120	0·00	15·31			
	77·75	31·30	15·99	13·07	1·2
180	0·00	22·32			
	78·61	47·73	25·41	21·60	1·2
			Theoretical ratio for complete combustion		3·5

Table 3. *Oxygen consumption and carbon dioxide production by washed suspensions of mycobacteria*

(Medium: 3·3 ml. 0·03 M-phosphate buffer, pH 7·4, at 38°. Total CO_2 (free and bound) measured by the method of Warburg & Yabusoe (1924).)

Glycerol (0·02 M)	Oxygen uptake (μl.)	Total CO_2 (μl.)	CO_2/O_2	'Extra' CO_2 / 'Extra' O_2
	Myco. smegmatis (11·0 mg. dry wt./cup; time 120 min.)			
Absent	220	215	0·98	—
Present	447	387	—	0·76
	Myco. butyricum (15·0 mg. dry wt./cup: time 75 min.)			
Absent	203	183	0·90	—
Present	392	308	—	0·66
			Theoretical R.Q.	0·86

Table 4. *Effect of reagents on the oxidation of glycerol and other substrates by mycobacteria*

(Washed suspensions (10–18 mg. dry wt. bacteria) in 3·5 ml. 0·03 M-phosphate buffer, pH 7·4. Gas, air. Time 60–120 min. in different experiments.)

	Q_{O_2}					
	Myco. smegmatis Substrate		*Myco. stercoris* Substrate		*Myco. butyricum* Substrate	
	Absent	Present	Absent	Present	Absent	Present
	Substrate, glycerol (0·02 M)					
Nil	11·3	28·1	12·6	26·8	15·6	26·6
Arsenite ($0·5 \times 10^{-2}$ M)	6·4	10·9	7·9	10·8	6·7	12·3
Arsenite ($1·0 \times 10^{-2}$ M)	6·1	10·8	—	—	6·3	10·5
Nil	11·3	28·1	10·5	20·5	12·1	29·9
Iodoacetate ($1·0 \times 10^{-3}$ M)	11·8	17·4	9·9	13·5	7·6	9·7
Nil	9·3	21·3	8·4	11·8	14·9	27·3
HCN ($1·0 \times 10^{-3}$ M)	7·5	8·9	4·3	4·9	5·0	5·2
HCN ($1·0 \times 10^{-2}$ M)	3·5	3·6	—	—	3·4	3·5
Nil	14·8	31·2	10·5	20·5	12·1	29·9
Malonate ($1·0 \times 10^{-2}$ M)	15·0	32·3	11·0	19·4	11·3	31·8
	Substrate, glyceraldehyde (0·02 M)					
Nil	11·3	41·7	—	—	15·6	24·2
Arsenite ($0·5 \times 10^{-2}$ M)	6·4	19·3	—	—	6·7	7·6
Arsenite ($1·0 \times 10^{-2}$ M)	6·1	19·6	—	—	6·3	6·6
	Substrate, pyruvate (0·02 M)					
Nil	15·9	37·6	—	—	11·8	27·2
Arsenite ($0·5 \times 10^{-2}$ M)	6·9	8·4	—	—	5·3	7·1
Arsenite ($1·0 \times 10^{-2}$ M)	6·8	8·5	—	—	5·3	7·2
						21·2

Table 5. *Influence of fluoride and fluoroacetate on the oxidation of glycerol, pyruvate and acetate by mycobacteria*

(Washed suspensions (13–17 mg. dry wt. bacteria) in 3·5 ml. 0·03 M-phosphate buffer, pH's 7·4 and 6·0. Gas, air. Time 60–120 min. in different experiments.)

	Q_{O_2}							
	Myco. smegmatis				*Myco. butyricum*			
	pH 7·4 Substrate		pH 6·0 Substrate		pH 7·4 Substrate		pH 6·0 Substrate	
Inhibitor (0·01 M)	Absent	Present	Absent	Present	Absent	Present	Absent	Present
	Substrate, glycerol (0·02 M)							
Nil	14·5	37·2	14·4	34·3	17·8	19·7	13·6	21·2
NaF	15·3	36·8	10·0	11·2	18·4	20·4	11·7	19·2
Nil	16·9	31·6	10·7	31·6	16·9	24·2	14·6	19·6
$CH_2FCOONa$	18·3	37·5	16·2	36·2	10·2	12·9	7·9	8·2
	Substrate, pyruvate (0·02 M)							
Nil	13·5	33·5	10·0	33·7	20·2	35·2	17·8	33·0
NaF	14·1	35·4	8·8	26·9	21·0	35·6	18·4	38·9
Nil	16·9	37·5	10·7	31·4	16·9	29·6	14·6	29·7
$CH_2FCOONa$	18·3	32·6	16·2	31·6	10·2	11·6	7·9	10·8
	Substrate, acetate (0·02 M)							
Nil	15·0	29·4	13·5	21·3	16·5	46·2	13·6	39·8
NaF	14·8	32·8	9·8	12·7	16·8	47·0	11·7	32·4
Nil	16·9	37·7	10·7	22·9	16·9	37·7	14·6	31·1
$CH_2FCOONa$	18·3	31·0	16·2	23·4	10·2	9·8	7·9	6·7

Products of oxidation

The fact that the Q_{O_2} values for the oxidation of pyruvate and glycerol are approximately equal (Table 1) is consistent with the suggestion that pyruvate is an intermediate in the aerobic breakdown of glycerol. If this is the case, pyruvate should accumulate when glycerol is oxidized in the presence of arsenite which inhibits pyruvate metabolism (Table 4). The Rothera test was applied to the contents of Warburg flasks (Table 4) after the bacteria and soluble protein had been removed by addition of 0·1 vol. of 30% (w/v) trichloroacetic acid. Pyruvate (blue ring) accumulated when glycerol or glyceraldehyde was metabolized in the presence, but not in the absence, of arsenite. The test was negative when the metabolism of glycerol was blocked by fluoride or iodoacetate (Table 5). These facts are consistent with the working hypothesis stated above.

Although the endogenous respiration is inhibited by arsenite, pyruvate did not accumulate in heavy suspensions of *Myco. smegmatis*; on the other hand, small amounts of pyruvic acid and traces of α-ketoglutaric acid (identified chromatographically (Cavallini, Frontali & Toschi, 1949) after crystallization of the 2:4-dinitrophenylhydrazones) were formed endogenously by *Myco. butyricum* respiring in the presence of arsenite. This difference is reflected in the cultural behaviour of the two organisms (Hunter, 1952). In washed suspensions of both species the yield of pyruvic acid was considerably increased when glycerol was metabolized in the presence of arsenite.

Experiments with bacterial extracts

Clear cell-free extracts of the bacteria were used to elucidate the intermediate steps in the breakdown of glycerol to pyruvic acid.

Anaerobic reduction of methylene blue. The possibility of glycerophosphate being an intermediate was tested by the Thunberg technique with methylene blue as hydrogen acceptor. Table 6 shows that dialysed extracts of *Myco. smegmatis* and *Myco. butyricum* buffered with inorganic phosphate do not oxidize glycerol unless ATP is added, suggesting that the first step in glycerol metabolism is a phosphorylation. This notion is supported by the fact that α-glycerophosphate is oxidized anaerobically in the absence of ATP (Table 6). In some extracts subjected to prolonged dialysis the oxidation of α-glycerophosphate was dependent on the addition of magnesium ions but the effect was not observed regularly. Control extracts which had been heated in a water bath for 15 min. at 100° did not reduce methylene blue in the presence or absence of substrates.

Since α-glycerophosphate is probably oxidized to triose phosphate, an attempt was made to detect triose phosphate by the sensitive method of Sibley & Lehninger (1949). At the end of an experiment the solutions were deproteinized with trichloroacetic acid and the methylene blue removed by filtration with charcoal. Several brands of charcoal produced precipitates which interfered with the colour test, but iron-free 'Norite A' (Pfanstiehl Chemical Co.) and Merck's medicinal charcoal gave satisfactory clear filtrates. The test reagents were added to the

Table 6. *Anaerobic triose phosphate formation from glycerophosphate and glycerol*

(Evacuated Thunberg tubes contained 2·0 ml. 0·1 M-phosphate buffer, pH 7·4 and 0·5 ml. methylene blue (1:1000) at 38°.)

Additions (ml.)	Dialysed cell-free extracts from *Myco. smegmatis*					*Myco. butyricum*			
Extract	1·0	1·0	1·0	1·0	1·0	0·2	0·2	0·2	0·2
α-Glycerophosphate (0·1 M)	0·3	0·3	—	—	—	0·3	0·3	—	—
Neutralized hydrazine hydrochloride (M)	—	0·2	0·2	—	0·2	—	0·2	0·2	0·2
Glycerol (0·2 M)	—	—	0·3	0·3	0·3	—	—	0·3	0·3
ATP (0·04 M)	—	—	—	0·2	0·2	—	—	—	0·2
Water	0·2	—	0·2	0·2	—	0·2	—	0·2	—
Reduction time (min.)	60	55	>300	100	75	>300	85	>300	65
Sibley-Lehninger Test	N.	P.	N.	N.	P.	N.	P.	N.	P.

N. = negative; P. = positive.

Table 7. *Oxygen consumption of dialysed extracts from* Mycobacterium smegmatis

(Each Warburg cup contained 2·5 ml. 0·03 M-phosphate buffer, pH 7·4. Temp., 38°. Gas, air. Time, 100 min. 1 ml. bacterial extract added from side bulb after equilibration.)

Additions	Oxygen uptake (μl.)	Sibley-Lehninger test
Nil	15	Negative
Neutralized hydrazine hydrochloride (0·05 M)	23	Negative
Methylene blue (0·003 %)	20	Negative
Hydrazine (0·05 M); methylene blue (0·003 %, w/v)	28	Negative
α-Glycerophosphate (0·01 M)	24	Negative
α-Glycerophosphate (0·01 M); diphosphopyridine nucleotide (0·1 mg., 50 % purity)	14	—
α-Glycerophosphate (0·01 M); hydrazine (0·05 M)	33	Trace
α-Glycerophosphate (0·01 M); methylene blue (0·003 %, w/v)	80	Negative
α-Glycerophosphate (0·01 M); methylene blue (0·003 %, w/v); diphosphopyridine nucleotide (0·1 mg. 50 % purity)	77	—
α-Glycerophosphate (0·01 M); methylene blue (0·003 %, w/v); hydrazine (0·05 M)	90	Positive

water-clear filtrates. When glycerol was the substrate, it was necessary to keep the concentration of magnesium ions to a minimum since precipitation of the hydroxide occurred on making the solution alkaline in the final stages of the Sibley-Lehninger test. Table 6 shows the anaerobic formation of triose phosphate from glycerol and glycerophosphate, the triose ester accumulating only when hydrazine was used as a fixative. The colour test is not specific for triose phosphate but is also given by ketonic acids. Since the nitroprusside test for pyruvate was negative, it is probable that the positive Sibley-Lehninger tests indicated the presence of triose phosphate.

Aerobic oxidation of glycerophosphate with methylene blue as hydrogen carrier. Dialysed extracts showed a small spontaneous oxygen uptake in the absence of added substrate. This blank was slightly increased by α-glycerophosphate (Table 7), but when methylene blue was added the oxygen consumption was increased fourfold. When the substrate was oxidized in the presence of hydrazine, triose phosphate was detectable by the Sibley-Lehninger test applied to the fluid from the manometric cups (after deproteinization and removal of methylene blue). The slow rate of oxygen consumption was not accelerated by the addition of diphosphopyridine nucleotide (Table 7).

When the experiments were repeated on a larger scale, the triose phosphate was determined as alkali-labile phosphate in deproteinized filtrates from which the methylene blue was removed by adsorption on charcoal as before (Table 8). Again it will be seen that triose phosphate accumulates only when the artificial hydrogen carrier is present and hydrazine is used as a fixative. Control experiments with boiled bacterial extracts and the requisite additions gave no alkali-labile phosphate; and analytical controls showed that hydrazine did not interfere with the Fiske and Subbarow phosphate determinations. It will be noticed (Table 8) that free inorganic phosphate increased during the experiment, especially in the presence of hydrazine. Since this did not occur with boiled extracts, the effect is attributed to phosphatase action.

Breakdown of phosphoenolpyruvate and D-3-*phosphoglycerate.* Dialysed extracts of *Myco. smegmatis* and *Myco. butyricum* are capable of dephosphorylating phosphoenolpyruvic acid (Table 9). The breakdown of D-3-phosphoglycerate was studied

Table 8. *Formation of triose phosphate from glycerophosphate*

(Clarified and dialysed bacterial extract (4 ml.) shaken in stoppered 50 ml. conical flasks for 3 hr. at 38°, unbuffered initial pH 6·8.)

Additions (total volume made up to 6 ml. with distilled water)	*Myco. smegmatis*		*Myco. butyricum*	
	Inorganic P	Alkali-labile P	Inorganic P	Alkali-labile P
	(μmoles/flask)			
Nil	0·3	0·0	5·8	0·3
1 ml. 0·1 M-α-glycerophosphate (100 μmoles)	3·2	2·3	6·7	1·3
1 ml. 0·1 M-α-glycerophosphate (100 μmoles) + 0·4 ml. 1·0 M-hydrazine hydrochloride (neutralized)	8·7	2·3	11·9	2·3
1 ml. 0·1 M-α-glycerophosphate (100 μmoles) + 0·5 ml. 1:200 methylene blue	3·8	2·9	7·1	0·3
1 ml. 0·1 M-α-glycerophosphate (100 μmoles) + 0·4 ml. 1·0 M-hydrazine + 0·5 ml. methylene blue	8·8	23·2	10·0	11·6

in the presence and absence of fluoride by measuring simultaneously the formation of phosphoenolpyruvate and the release of inorganic phosphate and pyruvic acid (Table 10). The degradation of phosphoglycerate does not occur unless magnesium ions are added and it is inhibited by fluoride, showing that the enzyme concerned has the characteristic properties of enolase. The amount

Table 9. *Enzymic cleavage of phosphopyruvate*

(4·0 ml. dialysed extract and 1·0 ml. 0·1 M-phosphoenolpyruvate (100 μmoles) incubated in stoppered 50 ml. conical flasks for 3 hr. at 38°, pH 6·8.)

	Myco. smegmatis (μmoles/flask)	*Myco. butyricum* (μmoles/flask)
Initial inorganic P	0·0	0·0
Final inorganic P	3·5	2·2
Pyruvic acid	2·8	1·9

of phosphoenolpyruvate accumulating is small in relation to the amount of pyruvate liberated. Control experiments showed that the disappearance of pyruvate was not significant under the conditions used. The activity of extracts was variable.

Phosphorylation of glycerol. When neutral unbuffered extracts are incubated with ATP, phosphate is liberated, the enzymic hydrolysis being complete in 3 hr. with *Myco. smegmatis* but incomplete with *Myco. butyricum*. Spontaneous liberation of phosphate was negligible when ATP was incubated with heat-inactivated extracts. Although the amount of inorganic phosphate set free is substantially reduced when glycerol is added to extracts of *Myco. smegmatis*, the residual ATP (7-min. acid-labile P) is negligible (Table 11). The change in the distribution of phosphate can be explained if glycerophosphate is formed. The esterification of glycerol is slower in extracts of *Myco. butyricum*. This interpretation of ATP breakdown is consistent with the observations which show that glycerophosphate is oxidized in extracts, whereas glycerol is not oxidized unless ATP is added (Table 6). Since the transphosphorylation experiments were performed in the absence of methylene blue, significant amounts of triose phosphate were not formed (Tables 7 and 8). Hence triose ester did not interfere with the determination of residual 7-min. acid-labile P. The inorganic phosphate content of the bacterial extracts was too small to be determined by the method used.

Table 10. *Enzymic breakdown of phosphoglycerate*

(Dialysed extract and substrate incubated in stoppered 50 ml. conical flasks for 3 hr. at 38°, pH 6·8. A control experiment without $MgSO_4$ gave zero results for all three types of P measured and for pyruvic acid.)

Contents (ml.)	Flask 1		Flask 2	
D-3-Phosphoglycerate 100 μmoles/ml.	1·0		1·0	
Bacterial extract	4·0		4·0	
$MgSO_4$ (0·04 M)	0·1		0·1	
NaF (0·2 M)	—		0·5	
Water	0·5		—	
	Myco. smegmatis (μmoles/flask)	*Myco. butyricum* (μmoles/flask)	*Myco. smegmatis* (μmoles/flask)	*Myco. butyricum* (μmoles/flask)
Initial inorganic P	0·0	0·0	0·0	0·0
Final inorganic P	46·0	9·7	4·5	0·0
Phosphopyruvate P	2·3	2·6	0·1	0·0
Pyruvic acid	42·0	9·6	0·1	0·0

Table 11. *Phosphorylation of glycerol*

(Reaction mixtures in 50 ml. conical flasks for 3 hr. at 38°.)

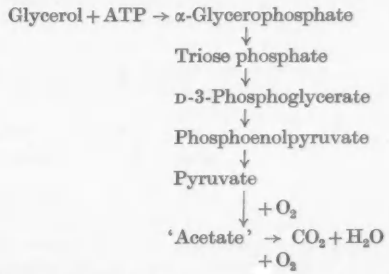
Contents (ml.)	Flask 1		Flask 2	
Glycerol (200 μmoles/ml.)	—		1·0	
Bacterial extract	4·0		4·0	
ATP (53·2 μmoles acid-labile 7-min. P/ml.)	0·4		0·4	
Water	1·0		—	
	Myco. smegmatis	*Myco. butyricum*	*Myco. smegmatis*	*Myco. butyricum*
	(μmoles/flask)		(μmoles/flask)	
Initial inorganic P	1·6	1·6	1·6	1·6
Final inorganic P	22·9	16·1	11·6	9·7
Final acid-labile 7-min. P + inorganic P	22·9	22·9	12·6	18·4
Initial acid-labile 7-min. P	21·3	21·3	21·3	21·3
ATP disappearing (as acid labile 7-min. P)	21·3	14·5	20·3	12·6
Glycerophosphate P	0·0	0·0	10·3	4·5

DISCUSSION

Glycerol is readily metabolized by washed suspensions of the mycobacteria used in this investigation. The oxidation is incomplete and accompanied by assimilation (Fig. 1, Tables 2 and 3); and yet significant amounts of intermediate metabolites do not appear in the suspension medium. In these respects the metabolism of glycerol conforms to a pattern characteristic of the acid-fast bacteria.

Arsenite, which blocks the metabolism of pyruvate, interferes with glycerol oxidation (Table 4), reducing oxygen consumption by more than 70 % and causing accumulation of pyruvate in the surrounding medium. Iodoacetate and fluoride, which also inhibit respiration in the presence of glycerol, operate at different intermediate stages since pyruvate does not accumulate extracellularly under their influence (Tables 4 and 5). These facts are compatible with the view that glycerol is normally broken down to pyruvate by a mechanism which involves phosphorylation; and they are not inconsistent with the meagre increase in oxygen uptake which occurs in the presence of α-glycerophosphate and D-3-phosphoglycerate (Table 1), because these substrates are known to penetrate cells with difficulty. Dialysed extracts, free from cells and insoluble cell debris, can phosphorylate glycerol (Tables 6 and 11) in the presence of ATP and can break down certain phosphorylated intermediates of carbohydrate metabolism. The activity of these extracts is weak and somewhat variable and usually lower with *Myco. butyricum* than with *Myco. smegmatis*. The extracts contain an α-glycerophosphate dehydrogenase which oxidizes α-glycerophosphate to triose phosphate anaerobically with methylene blue as a hydrogen acceptor (Table 6) or aerobically with methylene blue as a hydrogen carrier (Table 7). Prolonged dialysis has not revealed the presence of a dissociable coenzyme for this reaction. The extracts possess an enolase (inhibited by fluoride) which converts D-3-phosphoglycerate to phosphoenolpyruvate (Table 10) and an enzyme which splits phosphoenolpyruvic acid into pyruvic acid and inorganic phosphate (Table 9).

Since triose phosphate dehydrogenase has been found in extracts of *Myco. smegmatis* (Faine, Whiteside & Edson, 1951), the following scheme is put forward to represent the catabolism of glycerol in that organism and probably in other mycobacteria:



Both pyruvate and acetate are readily oxidized by intact cells (Table 1). Thus the catabolism of glycerol converges on the chief oxidative machinery of the mycobacteria, the initial phosphorylation being analogous to that which occurs in suspensions of homogenized mammalian tissues (Kalckar, 1939; Lindberg, 1951). Since glyceraldehyde and dihydroxyacetone are also oxidized (Table 1) an obligatory phosphorylation similar to that occurring in kidney (Lindberg, 1951) may initiate the breakdown.

In the above scheme diversion to synthetic processes (assimilation) is likely to take place at the triose phosphate and 'acetate' stages.

SUMMARY

1. Glycerol is oxidized and assimilated by washed suspensions of *Mycobacterium smegmatis* and *Mycobacterium butyricum*.

2. The oxidation is completely inhibited by cyanide and partially inhibited by selective agents such as arsenite, iodoacetate and fluoride. Pyruvate accumulates extracellularly in the presence of arsenite only.

3. Study of dialysed extracts has shown that the primary step in the degradation of glycerol is phosphorylation with adenosine triphosphate. Glycerophosphate is subsequently oxidized to triose phosphate and broken down to pyruvate by reactions of the Embden-Meyerhof scheme.

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REFERENCES

Baudran, G. (1910). *C.R. Acad. Sci., Paris*, **150**, 1200.
Bloch, H. (1944). *Schweiz. Z. Path.* **7**, 589.
Bloch, H., Matter, E. & Suter, E. (1947). *Amer. Rev. Tuberc.* **55**, 540.
Booth, V. H. & Green, D. E. (1938). *Biochem. J.* **32**, 855.
Cavallini, D., Frontali, N. & Toschi, G. (1949). *Nature, Lond.*, **163**, 568.
Dingle, J. H. & Weinzirl, J. (1932). *J. Bact.* **23**, 281.
Edson, N. L. (1951). *Bact. Rev.* **15**, 147.
Edson, N. L. & Hunter, G. J. E. (1943). *Biochem. J.* **37**, 563.
Edson, N. L. & Hunter, G. J. E. (1947). *Biochem. J.* **41**, 139.
Faine, S., Whiteside, M. & Edson, N. L. (1951). *Proc. Univ. Otago med. Sch.* **29**, 18.
Fiske, C. H. & Subbarrow, Y. (1925). *J. biol. Chem.* **66**, 375.
Fitzgerald, R. J. & Bernheim, F. (1948). *J. Bact.* **55**, 677.
Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
Friedemann, T. E. & Seibert, F. B. (1939). *Proc. Soc. exp. Biol., N.Y.*, **42**, 41.
Hammerschlag, A. (1891). *Z. klin. Med.* **12**, 9.
Hunter, G. J. E. (1952). *Proc. Univ. Otago med. Sch.* **30**, 10.
Kalckar, H. (1939). *Enzymologia*, **6**, 209.
Krebs, H. A. (1935). *Biochem. J.* **29**, 1620.
LePage, G. A. (1947). *J. biol. Chem.* **168**, 623.
Lindberg, O. (1951). *Biochim. biophys. Acta*, **7**, 349.
Lindsay, M., O'Donnell, T. V. & Edson, N. L. (1950). *Biochem. J.* **46**, 248.
Lohmann, K. & Meyerhof, O. (1934). *Biochem. Z.* **273**, 60.
Merrill, M. H. (1930). *J. Bact.* **20**, 235.
Merrill, M. H. (1931*a*). *J. Bact.* **21**, 361.
Merrill, M. H. (1931*b*). *J. Bact.* **21**, 375.
Nakamura, T. (1938). *Tôhoku J. exp. Med.* **34**, 231.
Nocard, E. & Roux, E. (1887). *Ann. Inst. Pasteur*, **1**, 19.
Sibley, J. A. & Lehninger, A. L. (1949). *J. biol. Chem.* **177**, 859.
Voris, L., Ellis, G. & Maynard, L. A. (1940). *J. biol. Chem.* **133**, 491.
Warburg, O. & Yabusoe, M. (1924). *Biochem. Z.* **146**, 380.
Wedum, A. G. (1936). *J. Bact.* **32**, 599.

The Action of Trypsin on Polylysine

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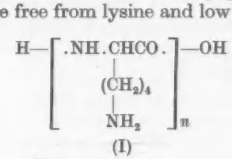
The way in which enzymes act is an outstanding problem in biochemistry, and of all substrates proteins are of predominating interest. Many investigations into the action of proteolytic enzymes have been carried out (Tiselius & Eriksson-Quensel, 1939; Linderstrøm-Lang & Jacobsen, 1941; Haugaard & Roberts, 1942; Winnick, 1944; Christensen, 1949; Desnuelle, Rovery & Bonjour, 1950; Rovery, Desnuelle & Bonjour, 1950; Smith, 1951), but it is a formidable task to elucidate the nature of all the products formed and even more difficult to measure their individual rates of formation. The kinetics of the hydrolysis of simple substrates catalysed by crystalline enzymes (particularly those from the pancreas, Neurath & Schwert, 1950) have been much studied and are relatively well understood. In general, such a substrate contains only one bond capable of being hydrolysed in the presence of the enzyme. Thus the problem of the relative rates of hydrolysis of several susceptible bonds in one substrate molecule does not arise. Synthetic polypeptides containing only one amino acid residue may have many susceptible bonds, but their relatively simple constitution restricts the number of products formed. Even here, the question whether all the susceptible bonds are split at the same rate, or some much faster than others, is not readily answered. This paper describes the attempt to answer this question in one case. The customary methods of following the rate of enzymic hydrolyses by measuring the overall rate of peptide-bond splitting are not directly helpful and the newer

chromatographic techniques have to be employed as well.

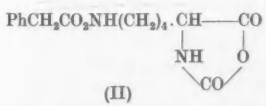
Katchalski (1951) found that polylysine (I) was readily attacked by crystalline trypsin. Estimation of the amino nitrogen (to determine the total extent of hydrolysis) and the carboxyl nitrogen (to determine the amount of amino acid formed) showed that about half the peptide bonds were split and practically no free lysine was formed. We have found by paper chromatography that the main products are dilysine (I; $n=2$) and trilysine (I; $n=3$). L-Lysine was used throughout this work. Apparently peptide bonds adjacent to a carboxyl or amino group are not rapidly split. Thus the nature of the terminal groups in the polymer may be of importance.

METHODS

The polypeptides used in this work were prepared by polymerization of the *N*-carboxyanhydride (II) followed by removal of the benzyloxycarbonyl groups by reduction (Katchalski, Grossfeld & Frankel, 1948). Water was used as initiator so that the polymers possessed terminal carboxyl groups (cf. Stahmann, Graf, Patterson, Walker & Watson, 1951); the products were shown by paper chromatography to be free from lysine and low peptides.



(I)



(II)

In a detailed examination of the kinetics of the action of trypsin on polylysines several of the customary methods for following the hydrolysis of peptides (Neurath & Schwert, 1950) were investigated, but with little success. We then found that the reaction could conveniently be followed by adding alkali to maintain the pH at a constant value.

The relationship between the extent of splitting and the amount of added alkali is obtained as follows: consider the splitting of an isolated peptide bond

$$—CO—NH— + H_2O \rightarrow —CO_2H + —NH_2.$$

Let the ionization constants of the carboxyl and ammonium groups be K_A and K_B. If

$$[—CO_2H] + [—CO_2^-] = [—NH_2] + [NH_3^+] = p,$$

then after the addition of sodium hydroxide the hydrogen-ion concentration will be given by

$$K_A p/(h+K_A) + [OH^-] = ph/(K_B+h) + h + [Na^+].$$

where h is the concentration of H^+. When the pH is near to 7, this becomes

$$[Na^+]/p = K_A/(h+K_A) - h/(h+K_B).$$

Since, in peptides $pK_A \simeq 3$, $K_A \gg [h]$, i.e.

$$[Na^+]/p = K_B/(K_B+h). \quad (1)$$

Consider the hydrolysis of a peptide (whose free α-ammonium group has an ionization constant K_1) to two products. Let the ionization constants of the free α-ammonium groups of these two products be K_2 and K_3, the initial concentration of the original peptide be x and the concentration of the products at any time be p. The amount of alkali required to bring the solution of the original peptide to a hydrogen-ion concentration h (near neutrality) will be $K_1 x/(K_1+h)$. The amount of alkali required to bring both the substrate left and the products formed to the hydrogen-ion concentration h is then

$$K_1(x-p)/(K_1+h) + K_2 p/(K_2+h) + K_3 p/(K_3+h).$$

Thus the amount of alkali required to keep the hydrogen-ion concentration equal to h during the hydrolysis is the difference between these two amounts, i.e.

$$[Na^+] = p[K_2/(K_2+h) + K_3/(K_3+h) - K_1/(K_1+h)]. \quad (2)$$

Thus the amount of alkali taken up is proportional to the number of peptide bonds split. If there is only one susceptible bond, the alkali uptake when all the substrate has been decomposed will give the constant of proportionality. But when there are several susceptible bonds the proportions of the different products formed and their ionization constants have to be known.

The ε-amino groups in lysine derivatives are strongly enough basic to be completely ionized at the pH at which the enzymic reactions were carried out, and so do not affect the amount of alkali taken up during the reaction.

The enzymic hydrolysis of esters has previously been followed by this method (Glick, 1937; Hawes & Alles, 1940; Schwert, Neurath, Kaufman & Snoke, 1948; Schønheyder, 1952), but it has not been used to study the hydrolysis of peptides. It is more convenient than the methods in which samples have to be withdrawn, and is well adapted to the use of small quantities of materials.

The enzymic hydrolysis was also followed by quantitative paper chromatography. Although laborious, this method is particularly valuable when the substrate contains several susceptible bonds because the rates of formation of the individual products can be measured.

EXPERIMENTAL

Materials

Polybenzyloxycarbonyllysines. ε-*N*-Benzyloxycarbonyl-*N*-carboxylysine anhydride (II) was polymerized in water-saturated nitrobenzene at 50°; the polymer was precipitated with light petroleum. The degrees of polymerization were estimated by amino-N (Van Slyke) analyses, and by titration in glacial acetic acid (Hall & Conant, 1927; Seaman & Allen, 1951); the two methods gave concordant results. The abbreviation '-mer' is used for the degree of polymerization, e.g. 230-mer.

Polylysine hydriodides. Reduction of the polybenzyloxycarbonyllysines was carried out with phosphonium iodide in acetic acid; the hydriodides did not move from the original position on paper chromatograms using *n*-butanol:acetic acid:water:pyridine (30:6:24:20, v/v).

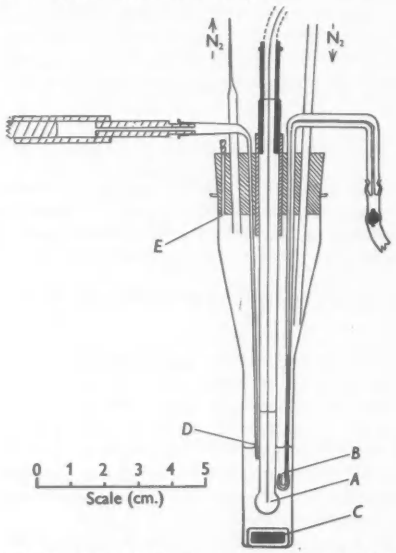


Fig. 1. Potentiometric titration apparatus used for following enzymic hydrolyses. *A*, glass electrode; *B*, calomel electrode; *C*, glass-enclosed iron rod for magnetic stirring; *D*, fine glass tube connected to Agla micrometer syringe; *E*, hole for introduction of enzyme.

Peptides of lysine. The preparation of di-, tri-, tetra- and penta-lysine is described elsewhere (Waley & Watson, 1953). Hexa-, hepta- and octa-lysine were isolated from an acid hydrolysate of polylysine by chromatography on paper. This procedure yielded chromatographically homogeneous samples in sufficient amount for enzymic experiments.

Trypsin. Crystalline trypsin (50% $MgSO_4$) from Armour Laboratories (Charterhouse Street, London, E.C. 1) was used; a stock solution in 0·01 M-$CaCl_2$ kept at 2° retained its full activity for at least a week.

Apparatus

The reaction vessel is shown in Fig. 1. CO_2 was excluded by passing N_2 (previously saturated with water at 25·8°) over the surface of the solution in the vessel, which was in a thermostat at 25·8°. The volume of the solution was 2·75 ml., and was not significantly altered by the alkali added. Initially the solution was brought to pH 7·60; subsequently, the pH dial of the pH meter was left unaltered. The enzyme solution was added, and enough alkali to give a galvanometer deflexion of about one division (a pH change of about 0·04); the time was noted when the deflexion fell to zero, further alkali added and so on.

RESULTS

Chromatographic experiments

Qualitative. The nature of the products obtained in the enzyme-catalysed hydrolysis of polymer, with pH maintained at 7·6 by the addition of alkali, is shown in Pl. 3 (*a*) (20·4 mg. polylysine hydriodide (230-mer)/ml.; $4·3 \times 10^{-3}$ mg. trypsin N/ml.; potassium chloride, 0·083 M). The reaction can be divided into two phases: in the first, as the polymer disappeared the major products were di-, tri- and tetra-lysine; in the second phase, as tetralysine disappeared the main products were di- and trilysine, together with some lysine (the decomposition of tetralysine has been found to give rise to some lysine). Pl. 3 (*b*) shows the course of an acid-catalysed hydrolysis (20·8 mg. polylysine hydriodide/ml. in constant boiling-point aqueous HBr at 25·8°). Comparable amounts of all the peptides were formed in the early stages, in contrast to the enzymic reaction.

The reactivities towards trypsin of tetra-, penta-, hexa-, hepta- and octa-lysine were compared in the following way. Polylysine hydriodide (330 mg.) in constant boiling-point HBr (15 ml.) was kept at 25·8° for 4 days; the solution was freed from the bulk of HBr by distillation, followed by repeatedly adding water and evaporating. The residue (containing a range of lysine peptides, Pl. 3 (*b*)) was dissolved in water (5 ml.), and 0·5 ml. was fractionated by chromatography on two Whatman 3MM papers, 12 × 8 in. (30·5 × 20·3 cm.), applying a band along the starting line. Synthetic tetralysine was used as a marker. After development with the butanol:acetic acid:pyridine:water solvent for 5 days, the papers were dried at room temperature, heated at 100° for 15 min., and the positions of the bands revealed by their fluorescence in ultraviolet light. The strips of paper containing tetra-, penta-, hexa-, hepta- and octa-lysine were cut out and eluted with 0·1 N-acetic acid, about 0·5 ml. being collected. The solutions were freeze-dried, water added, and the solutions freeze-dried again. The peptides were then dissolved in collidine buffer (pH 7·70) [collidine (Eastman Kodak), 11·6 ml.; 0·2 N-HCl, 140 ml.; water, 250 ml.] containing $8·8 \times 10^{-4}$ mg. trypsin N/ml. The solutions were kept at 25·8°, and at various times samples were transferred to a sheet of Whatman no. 1 paper. Drops of 2 N-acetic acid had previously been placed on the starting line so that the enzymic reaction did not continue on the paper. After development for 60 hr. with the solvent described above the spots were revealed with ninhydrin.

Examination of the intensities showed that while the tetralysine was largely intact after 160 min., all

(*a*) The action of trypsin on polylysine. Substrate concentration: 20·4 mg. polylysine hydriodide/ml.; enzyme concentration: $4{\cdot}3 \times 10^{-3}$ mg. enzyme N/ml. KCl, 0·083*M*. Temp., 25·8°. (*b*) The action of hydrobromic acid on polylysine. Substrate concentration: 20·8 mg. polylysine hydriodide/ml. in constant boiling-point HBr. Temperature, 25·8°. Solvent: *n*-butanol:acetic acid:water:pyridine (30:6:24:20, v/v).

the higher peptides were about half decomposed after this time. Investigation of the rates of decomposition of tetra- and tri-lysine using higher trypsin concentrations showed that trilysine was about a 100 times less reactive than tetralysine, which was about 10 times less reactive than pentalysine. Dilysine was completely unreactive.

Quantitative. In this experiment the rate of addition of alkali to maintain the pH at 7·6 was measured (thus giving a measure of the overall rate of bond splitting), and samples were withdrawn periodically to measure the rate of formation of the individual products by paper chromatography.

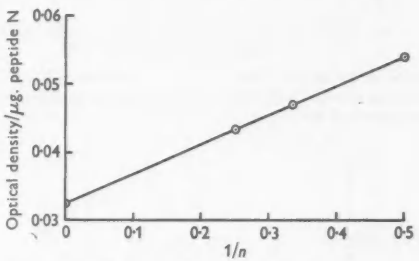
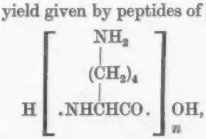


Fig. 2. Colour yield given by peptides of lysine,



in the ninhydrin reaction.

The reaction mixture was 21 mg. polylysine hydriodide/ml.; $2·1 \times 10^{-3}$ mg. trypsin N/ml.; KCl, 0·083 M. The samples (11·3 μl.) were withdrawn and transferred to a sheet of Whatman no. 1 paper; the reaction was stopped with acetic acid as described above. Known amounts of dilysine, trilysine, tetralysine and the polymer were also included on the chromatogram for calibration. After development for 64 hr. with butanol:acetic acid:water:pyridine (30:6:24:20), the colorimetric estimations were carried out as described by Waley & Watson (1953).

There is a linear relationship between the colour yield/mg. peptide N and the reciprocal of the number of lysine residues in the peptide (Fig. 2), from which the colour yield for pentalysine was obtained by interpolation. The results are given in Table 1, and are shown, calculated in molar terms, in Figs. 3 and 4.

The total nitrogen present in 11·3 μl. calculated from a nitrogen estimation was 24·9 μg.; the total found (Table 1) was usually lower than this because neither material between the polymer and pentalysine spots (consisting of hexa- to nona-lysines) nor lysine (formed only from tetralysine), was estimated.

Kinetic results obtained by titration

Enzymic hydrolysis of polylysine. In all the measurements of rates of polymer decomposition the enzyme concentration was 1·6 μg. trypsin N/ml. and except when otherwise stated the runs were conducted in 0·083 M-potassium chloride, to ensure an effectively constant ionic strength.

A typical plot of the alkali uptake is shown in Fig. 5; the reaction is zero order throughout a considerable part of its course. When the initial rate of alkali uptake (v) was plotted against the initial substrate concentration (s), a curve of the form commonly obtained in enzyme-catalysed reactions was obtained, i.e.

$$v = aes/(b+s), \qquad (3)$$

where a and b are constants and e is the concentration of enzyme (Michaelis & Menten, 1913). The parameters a and b were found from the rearranged form:

$$s/v = b/ae + s/ae. \qquad (4)$$

This relationship was obeyed by all of the polymers studied (see Fig. 6). When the substrate concentration was expressed in moles/l. (this point is discussed later), and the enzyme concentration also expressed in moles/l. (taking the molecular weight of trypsin as 36 500), the values of a and b given in Table 2 were obtained.

Table 1. *The rate of enzymic hydrolysis of polylysine followed by quantitative paper chromatography*

(Substrate, 21 mg./ml.; trypsin, $2·1 \times 10^{-3}$ mg. N/ml., in 0·083 M-KCl. Amounts of peptides are expressed in μg. of N present in 11·3 μl.)

Time (sec. $\times 10^2$)	Dilysine	Trilysine	Tetralysine	Pentalysine	Polylysine	Total
3	0·10	0·50	0·72	0·27	24·6	26·2
5	0·50	1·00	0·72	0·74	20·4	23·4
8	1·08	1·92	1·80	0·34	17·7	22·8
11	1·68	3·32	2·80	0·66	14·6	23·1
17	2·75	4·50	3·70	1·32	9·9	22·2
21	3·65	6·30	4·51	1·03	6·2	21·7
30	4·57	8·84	5·04	1·05	2·4	21·9
48	6·36	9·20	5·90	0·01	0	21·5
72	6·55	10·15	5·26	0	0	22·0
182	8·38	9·83	2·83	0	0	21·0

The initial rate of the polymer decomposition was found to decrease when the ionic strength was lowered; thus, for the 70-mer, when the concentration of potassium chloride was reduced by a factor of 4 to 0·021 M, the constants a and b were 13·3 sec.$^{-1}$, and $5{\cdot}8 \times 10^{-4}$ moles/l.

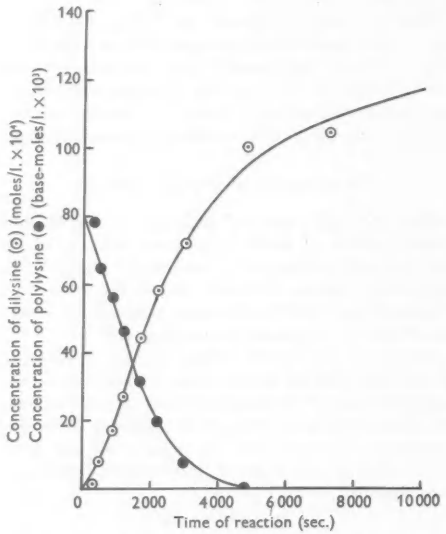


Fig. 3. Formation of dilysine (⊙), and decomposition of polylysine (●), followed by quantitative paper chromatography. Initial polylysine hydriodide, 21 mg./ml., trypsin, $2{\cdot}1 \times 10^{-3}$ mg. enzyme N/ml. KCl, 0·083 M.

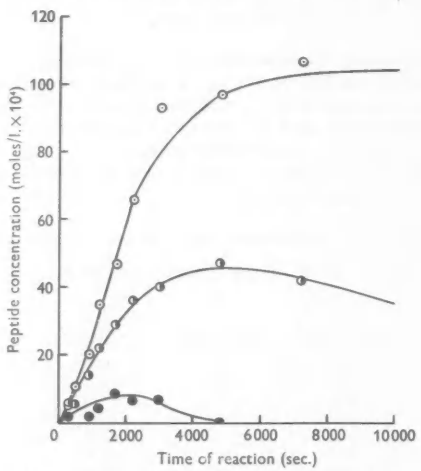


Fig. 4. Formation of trilysine (⊙), tetralysine (◑), and pentalysine (●), followed by quantitative paper chromatography. Conditions as described for Fig. 3.

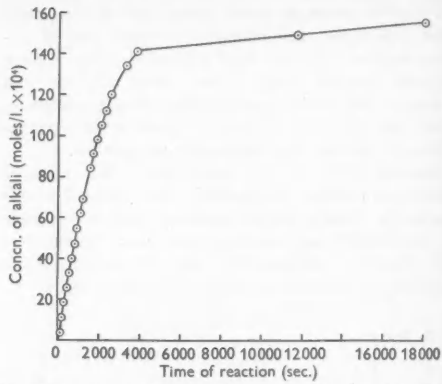


Fig. 5. Amount of alkali added to maintain pH at 7·6 during the enzymic hydrolysis of polylysine. Conditions as described for Fig. 3.

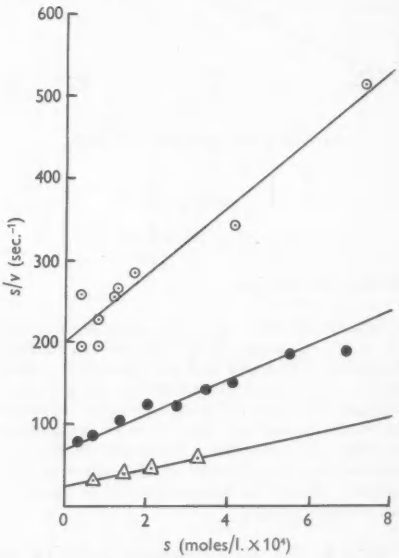


Fig. 6. Enzymic hydrolysis of polylysine; variation of s/v with s, where s is the molar polymer concentration and v is the initial rate of alkali consumption. ⊙, 19-mer; ●, 70-mer; △, 230-mer. Trypsin $1{\cdot}6 \times 10^{-3}$ mg. enzyme N/ml.

Table 2. *Values of the constants in the Michaelis-Menten equation (equation (3)) for polymers of differing degrees of polymerization*

Degree of polymerization of polylysine	a (sec.$^{-1}$)	b (moles/l. × 10^4)
19	9·4	4·8
70	18·6	3·1
230	36	2·0

Enzymic hydrolysis of tetralysine. Paper chromatography showed that the main product was dilysine, but that some lysine and trilysine were also formed. The kinetics of the hydrolysis were studied by following the uptake of alkali at pH 7·6 in 0·083 M-potassium chloride with 0·024 mg. trypsin N/ml. A straight line resulted when the initial rates were plotted according to equation (4) (Fig. 7); the values of a and b were 0·54 sec.$^{-1}$ and $1{\cdot}75 \times 10^{-3}$ moles/l. respectively.

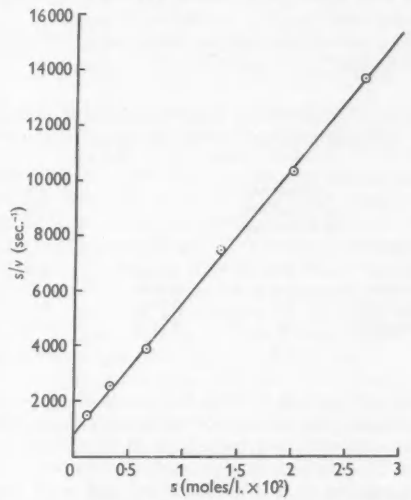


Fig. 7. Enzymic hydrolysis of tetralysine; variation of s/v with s, where s is the molar tetralysine concentration and v is the initial rate of alkali consumption. Trypsin $2{\cdot}4 \times 10^{-2}$ mg. enzyme N/ml.

Enzymic hydrolysis of pentalysine. Paper chromatography showed that the products in this case were di- and tri-lysine. The kinetics were followed as described for tetralysine, but using 0·4 μg. trypsin N/ml. The values of a and b obtained from the linear plot according to equation (4) (Fig. 8) were 12 sec.$^{-1}$ and $5{\cdot}6 \times 10^{-4}$ moles/l. respectively.

DISCUSSION

Some insight into the mode of action of trypsin on polylysine may be obtained from Pl. 3. While the polymer is undergoing decomposition no lysine is formed. Thus the terminal peptide bonds are not attacked. The main question is whether all the bonds, apart from the immune end bonds, are equally susceptible (random fission) or whether some bonds are broken much more readily than others (selective fission). The acid-catalysed hydrolysis of polylysine (Pl. 3) shows two important features: (*a*) lysine is formed; (*b*) comparable amounts of all the peptides are formed early in the course of the reaction. The acid-catalysed hydrolysis appears to be of the random-fission type. The trypsin-catalysed hydrolysis, on the other hand,

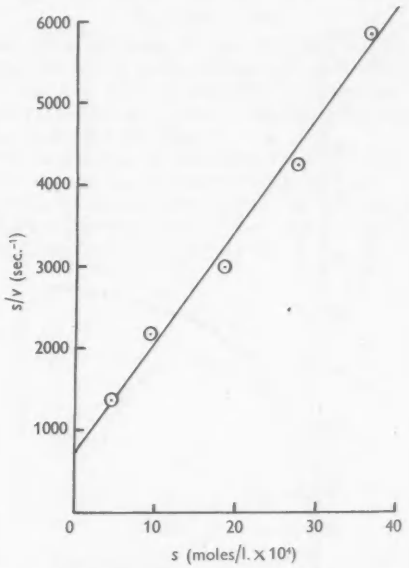


Fig. 8. Enzymic hydrolysis of pentalysine; variation of s/v with s, where s is the molar pentalysine concentration and v is the initial rate of alkali consumption. Trypsin 4×10^{-4} mg. enzyme N/ml.

produces initially mostly di-, tri- and tetra-lysine. This suggests that the bonds most readily split are those near the end of a chain unless the missing higher peptides (penta- to octa-lysine) are particularly reactive. If the velocity constants of decomposition of these peptides were much higher than those of their formation from polymer, their concentrations would always be very low. The chromatographic experiments described above, however, showed that the peptides (penta- to octa-lysine) were decomposed at roughly the same rate by trypsin, and (as is shown in Fig. 9) pentalysine is broken down about as fast as the polymers.

Susceptible bonds

...lysyl—lysyl—lysyl—lysyl—lysyl—lysyl—lysyl—lysyl—lysine

8 7 6 5 4 3 2 1

(III)

In considering in more detail the action of trypsin on the polymer one may start by examining the results obtained by paper chromatography (Table 1 and Figs. 3 and 4). Pl. 3 showed that the reaction could be divided into two phases and this is confirmed by Figs. 3 and 4; in the first phase, lasting about 5000 sec., the polymer is decomposed and di-, tri- and tetra-lysine are rapidly formed, while pentalysine is formed and then decomposes. In the second phase tetralysine slowly decomposes,

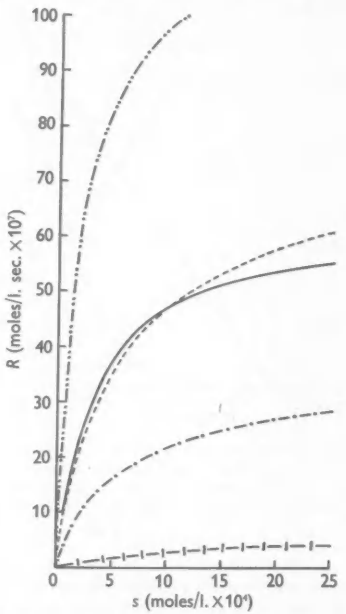


Fig. 9. Variation of initial rate of bond fission (R) with substrate concentration (s). –|–|–|, Tetralysine; - - - - -, pentalysine; –·–·–·–, polylysine (19-mer); ——, polylysine (70-mer); –··–··–··, polylysine (230-mer). Calculated for a trypsin concentration of $1{\cdot}6 \times 10^{-3}$ mg. enzyme N/ml.

mainly to dilysine, the amount of trilysine not changing appreciably. The first phase of this reaction was also followed by the titration method (Fig. 5). From the following argument the contribution of formation of di-, tri-, tetra- and penta-lysine to the total rate of alkali uptake is deduced. When a molecule of a small peptide (e.g. dilysine) is split from the large polymer chain the pK values of the original polymer (e.g. n-mer) and final polymer (n-2 mer) will not differ appreciably, so that the contribution of this step to the total amount of alkali consumed will be governed by the pK of the α-amino group of dilysine, i.e. equation (2) applies with $K_1 = K_3$. The initial rates of formation of the peptides are given in Table 3, together with the values of $K_2/(K_2+h)$ at pH 7·6, using the pK values given previously (Waley & Watson, 1953). Thus the rate of uptake of alkali due to the splitting off of each peptide can be calculated (Table 3, column 4). The initial rate of alkali uptake obtained from Fig. 5 was $6{\cdot}06 \times 10^{-6}$ moles/l. sec., so that the liberation of di-, tri-, tetra- and penta-lysine accounts for more than half this amount. Since the original polymer had over 200 peptide bonds, the main reaction is clearly the formation of the small peptides mentioned above: the question raised above, therefore, is answered in favour of selective fission.

Table 3. *Contribution of the individual steps in the polymer breakdown to the total alkali uptake*

Peptide	Initial rate of formation ($10^6 \times$ moles/l.sec.)	$K_2/(K_2+h)$	Contribution to alkali uptake ($10^6 \times$ moles/l.sec.)
Dilysine	1·2	0·523	0·63
Trilysine	1·7	0·686	1·17
Tetralysine	1·3	0·738	0·96
Pentalysine	0·8	0·772	0·62
	5·0		3·38

It follows that most of the bonds being broken during the enzymic hydrolysis, are those near one or both ends of the polymer chain. It may be assumed that the enzyme must be attached to the polymer in the vicinity of a given bond for this bond to be broken, since trypsin has one 'active centre' per molecule (Balls & Jansen, 1952). Assuming that the enzyme may combine with any residue and that the complex so formed can only break down in one way, one arrives at the following scheme:

$$x_n + E \underset{k_2'}{\overset{k_1'}{\rightleftarrows}} (x_n E)_I \xrightarrow{k_3'} x_{n-2} + x_2 + E \qquad (5)$$

$$x_n + E \underset{k_2''}{\overset{k_1''}{\rightleftarrows}} (x_n E)_{II} \xrightarrow{k_3''} x_{n-3} + x_3 + E, \qquad (6)$$

$$x_n + E \underset{k_2'''}{\overset{k_1'''}{\rightleftarrows}} (x_n E)_{III} \xrightarrow{k_3'''} x_{n-4} + x_4 + E, \qquad (7)$$

where x_n represents the polymer (degree of polymerization n) and E the trypsin. If the total enzyme concentration is E_0, and

$$K_m' = (k_2' + k_3')/k_1', \quad K_m'' = (k_2'' + k_3'')/k_1'', \text{ etc.},$$

then applying the stationary state method the equations have the form

$$\mathrm{d}[x_2]/\mathrm{d}t = k_3' E_0 [x_n]/K_m' \{1 + [x_n]\, \Sigma(1/K_m)\}, \qquad (8)$$

for the initial rates of formation of dilysine, trilysine, etc. The total initial rate of bond splitting (R) is then

$$R=[x_n]\,E_0\Sigma(k_3/K_m)/\{1+[x_n]\,\Sigma(1/K_m)\}, \quad (9)$$

where $\Sigma(1/K_m)=1/K'_m+1/K''_m+1/K'''_m+\ldots,$

and $\Sigma(k_3/K_m)=k'_3/K'_m+k''_3/K''_m+k'''_3/K'''_m+\ldots.$

The term 'initial rate' is used in the conventional sense; the initial rate of formation of products is, strictly, zero in any scheme in which the products are formed from a complex. As in other work with enzymes, the complex must have been formed very rapidly, since if the complex were only slowly formed the rate of formation of the products would at first increase and then decrease; in fact this rate decreases steadily. The use of the stationary state method in this connexion has been discussed elsewhere (Waley, 1953).

Equation (9) is of the same form as the empirically found equation (3), i.e. that of the Michaelis-Menten equation with a Michaelis constant of $1/\Sigma(1/K_m)$ and a specific rate for the decomposition of the enzyme-substrate complex of $\Sigma(k_3/K_m)/\Sigma(1/K_m)$. The relative rates of formation of the products are, from equation (8), independent of the initial concentration of the polymer; let it be assumed that they are also independent of its degree of polymerization in order to calculate the values of $\Sigma(1/K_m)$ and $\Sigma(k_3/K_m)$. From the data in Table 3 it can be shown that 0·76 mole of alkali is taken up for each mole of bonds split, so that the rates of alkali uptake (v) can be converted to rates of bond splitting (R), from which the values of $1/\Sigma(1/K_m)$ and $\Sigma(k_3/K_m)/\Sigma(1/K_m)$ given in Table 4 were obtained.

Table 4. *Values of the constants in the Michaelis-Menten equation (equation (9)) for tetra-, penta- and polylysines*

Substrate	$10^4/\Sigma(1/K_m)$ (moles/l.)	$\Sigma(k_3/K_m)/\Sigma(1/K_m)$ (sec.$^{-1}$)
Polylysine (19-mer)	4·8	12
Polylysine (70-mer)	3·1	24·5
Polylysine (230-mer)	2·0	47
Tetralysine	17·5	2·7
Pentalysine	5·6	30

On this scheme of polymer breakdown the relative rates of formation of the products are

$$\mathrm{d}[x_2]/\mathrm{d}t:\mathrm{d}[x_3]/\mathrm{d}t:\mathrm{d}[x_4]/\mathrm{d}t\ldots=k'_3/K'_m:k''_3/K''_m:k'''_3/K'''_m\ldots. \quad (10)$$

It has already been mentioned that the main reaction is the formation of di-, tri-, tetra- and pentalysine, so that the values of k_3/K_m must be lower for splitting in the middle of the chain. There are two extreme cases: either the enzyme combines preferentially with the residues near the end of the chain; or the enzyme combines equally readily with any residue, but only those complexes formed with residues near the end of the chain have specially labile bonds. In the latter case the residues in the middle of the chain would slow the reaction down, so that the overall rate of hydrolysis of the larger polymers would be slower than that of the smaller ones. In fact, this is not the case (Fig. 9). Thus the variation in the values of k_3/K_m referred to above must be caused by variations in k_1 or k_2.

Chromatographic experiments showed that tetralysine decomposed to lysine, dilysine and trilysine. When one mole of tetralysine was decomposed 0·20 mole of alkali was needed to maintain the pH at 7·6; if the overall reaction takes the course

$$3x_4 \rightarrow 4x_2+x_3+x,$$

then, using equation (2), the calculated alkali consumption is 0·19 mole of alkali per mole tetramer decomposed. Since the reaction is composite, the values given in Table 4 have the same significance as they do for the polymers.

When one mole of pentalysine was decomposed, 0·40 mole of alkali was needed to keep the pH at 7·6, and this factor was used to obtain the values of the constants given in Table 4; the value of the factor found from equation (2) was 0·44.

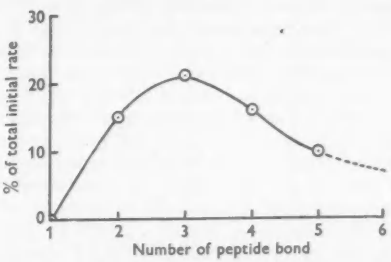


Fig. 10. Variation of reactivity of bonds with position in polymer chain; fission of bond no. 1 gives lysine, no. 2 gives dilysine, etc.

The initial rates of bond fission (for a trypsin concentration of $1{\cdot}6\times10^{-3}$ mg. enzyme nitrogen/ml.) for all the substrates examined are plotted against the substrate concentrations in Fig. 9. The curves were derived from the straight lines of Figs. 6–8 by conversion of the rates of alkali consumption to rates of bond fission.

Some insight into the reasons for the relative lability of the peptide bonds in polylysine in the trypsin-catalysed hydrolysis can be obtained from the initial rates in Table 3. In Fig. 10 these rates are expressed as percentages of the total initial rate plotted against the position of the peptide bond in the polymer chain. The shape of this curve can be accounted for by supposing that there are two opposing factors governing the relative lability of the bonds: (*a*) the nearer the bond is to the reactive group at the end of a chain, the more slowly does the enzyme attack it; (*b*) the further the bond is from the middle of the chain, the more rapidly does the

enzyme attack it. The existence of the first factor is well known; trypsin does not catalyse the hydrolysis of peptide bonds adjacent to an unmasked amino group (Neurath & Schwert, 1950) and the same appears to hold for carboxyl groups. It is presumably this factor which is responsible for the marked gradation in the susceptibility of the lower peptides of lysine. The existence of the second factor mentioned above is more surprising, and may be connected with a difference in configuration of the residues near the end of a chain. In the form of a film cast from aqueous solution polylysine hydriodide was shown to belong to the class of mainly α-folded polypeptides (A. Elliott, private communication), and it seems reasonable to suppose that the configuration is the same in aqueous solution. Now the end residues of a mainly α-folded polypeptide chain are believed to exist largely in the unfolded, β, configuration (C. H. Bamford & W. E. Hanby, private communication) since the ratio of the amount of β- to the amount of α-form increases with decreasing degree of polymerization (Bamford, Hanby & Happey, 1951). Thus the end residues in polylysine probably exist largely in the β-configuration. If, then, we assume that trypsin combines preferentially with residues in the β-configuration, the second factor referred to above is explained. This may be correlated with the fact that denatured proteins are, in general, much more susceptible to enzymic hydrolysis than native proteins, and furthermore that in some cases native proteins are predominantly in the α- and denatured proteins in the β-configuration (Ambrose & Elliott, 1951).

The rate of hydrolysis of polylysine (70-mer) was found to depend on the ionic strength. Reducing the ionic strength increased the Michaelis constant, i.e. the enzyme has less affinity for the substrate. This is probably connected with the extent to which the polymer chain is coiled, since the viscosities of polylysine were found to depend greatly on the nature of the medium in a way characteristic of poly-electrolytes (Fuoss, 1951).

It is interesting that both random fission and selective fission occur in the enzymic hydrolysis of polysaccharides. Attack of amylose by α-amylase apparently occurs by random fission (Meyer & Gonon, 1951; Roberts & Whelan, 1952) and here also end bonds are immune. The enzyme β-amylase, on the other hand, attacks amylose to give maltose as sole product, so that the fission is selective; also, during the course of the reaction, only amylose and maltose are present (Kerr & Cleveland, 1951) so that this is a particularly clear example of the 'explosion' theory sometimes suggested for the action of enzymes on proteins (Tiselius & Eriksson-Quensel, 1939; Winnick, 1944). The contrast between the modes of action of α- and β-amylase is shown clearly when maltohexaose is the substrate: with α-amylase, the products are three molecules of maltose and one of maltotriose (Roberts & Whelan, 1952): with β-amylase, only maltose is formed (Bailey, Whelan & Peat, 1950).

SUMMARY

1. The action of trypsin on peptides and polymers of lysine has been investigated. Chromatographic methods have been used both to identify the products formed and to follow their rate of formation. The rate of peptide-bond splitting has been estimated by measuring the amount of alkali required to maintain the pH constant during the enzymic hydrolysis.

2. The chief products from polylysine are dilysine and trilysine. The end bonds (next to a carboxyl or amino group) are not split, but those near the end are split preferentially, so that the main reaction initially is a stepwise formation of the lower peptides.

3. Pentalysine breaks down to dilysine and trilysine at a rate comparable with the rate of breakdown of the polymers. Tetralysine reacts more slowly, giving mostly dilysine; trilysine is much less reactive and dilysine is completely inert.

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REFERENCES

Ambrose, E. J. & Elliott, A. (1951). *Proc. Roy. Soc.* A, **208**, 75.
Bailey, J. M., Whelan, W. J. & Peat, S. (1950). *J. chem. Soc.* p. 3692.
Balls, A. K. & Jansen, E. F. (1952). *Advanc. Enzymol.* **13**, 321.
Bamford, C. H., Hanby, W. E. & Happey, F. (1951). *Proc. Roy. Soc.* A, **206**, 407.
Christensen, L. K. (1949). *Nature, Lond.*, **163**, 1003.
Desnuelle, P., Rovery, M. & Bonjour, G. (1950). *Biochim. biophys. Acta*, **5**, 116.
Fuoss, R. M. (1951). *Disc. Faraday Soc.* **11**, 125.
Glick, D. (1937). *Biochem. J.* **31**, 521.
Hall, N. F. & Conant, J. B. (1927). *J. Amer. chem. Soc.* **49**, 3047.
Haugaard, G. & Roberts, R. M. (1942). *J. Amer. chem. Soc.* **64**, 2664.
Hawes, R. C. & Alles, G. A. (1940). *J. biol. Chem.* **133**, 375.
Katchalski, E. (1951). *Advanc. protein Chem.* **6**, 123.
Katchalski, E., Grossfeld, I. & Frankel, M. (1948). *J. Amer. chem. Soc.* **70**, 2094.

Kerr, R. W. & Cleveland, F. C. (1951). *J. Amer. chem. Soc.* **73**, 2421.
Linderstrøm-Lang, K. & Jacobsen, C. F. (1941). *C.R. Lab. Carlsberg*, **24**, 1.
Meyer, K. H. & Gonon, W. F. (1951). *Helv. chim. acta*, **34**, 294.
Michaelis, L. & Menten, M. L. (1913). *Biochem. Z.* **49**, 333.
Neurath, H. & Schwert, G. W. (1950). *Chem. Rev.* **46**, 69.
Roberts, P. J. P. & Whelan, W. J. (1952). *Biochem. J.* **51**, xviii.
Rovery, M., Desnuelle, P. & Bonjour, G. (1950). *Biochim. biophys. Acta*, **6**, 166.
Schønheyder, F. (1952). *Biochem. J.* **50**, 378.
Schwert, G. W., Neurath, H., Kaufman, S. & Snoke, J. E. (1948). *J. biol. Chem.* **172**, 221.
Seaman, W. & Allen, E. (1951). *Analyt. Chem.* **23**, 592.
Smith, E. L. (1951). In *The Enzymes*, Ed. by Sumner, J. B. and Myrbäck, K. vol. 1, pt. II, p. 793. New York: Academic Press Inc.
Stahmann, M. A., Graf, L. H., Patterson, E. L., Walker, J. C. & Watson, D. W. (1951). *J. biol. Chem.* **189**, 45.
Tiselius, A. & Eriksson-Quensel, I. B. (1939). *Biochem. J.* **33**, 1752.
Waley, S. G. (1953). *Biochim. biophys. Acta*, **10**, 27.
Waley, S. G. & Watson, J. (1953). *J. chem. Soc.* p. 475.
Winnick, T. (1944). *J. biol. Chem.* **152**, 465.

Studies in Detoxication

54. THE METABOLISM OF BENZENE. (*a*) THE FORMATION OF PHENYLGLUCURONIDE AND PHENYLSULPHURIC ACID FROM [^{14}C]BENZENE. (*b*) THE METABOLISM OF [^{14}C]PHENOL

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Parke & Williams (1953) have shown, using [$^{14}C_1$]benzene, that phenol is a major metabolite of benzene in rabbits. This phenol, however, is not excreted as such but as the glucuronide and ethereal sulphate. Porteous & Williams (1949) have estimated the glucuronic acid and ethereal sulphate outputs of rabbits receiving benzene, but their results represent the conjugates of all the phenolic metabolites of benzene, that is, phenol, quinol, catechol and hydroxyquinol. The use of [^{14}C]-benzene has now enabled us to determine the amount of benzene excreted as phenylglucuronide and phenylsulphuric acid.

In the intact animal, benzene gives rise to *trans-trans*-muconic acid, and it is possible that phenol is an intermediate in the formation of this acid. To test this possibility ^{14}C-labelled phenol, obtained as a metabolite of benzene in our previous experiments (Parke & Williams, 1953), was used. This phenol, since it is obtained from [$^{14}C_1$]benzene, is presumably a mixture of four isomeric [^{14}C]phenols, namely [1-, 2-, 3- and 4-^{14}C]phenols. Using this phenol, it was also possible to find out how much administered phenol was excreted as glucuronide and ethereal sulphate and how much was oxidized to quinol and catechol. ^{14}C-Labelled phenylglucuronide was also prepared from the urine of rabbits receiving labelled phenol or benzene. With this compound the reliability of the usual systematic lead-acetate-precipitation procedure for the isolation of glucuronides from urine could be tested.

EXPERIMENTAL

Measurement of radioactivity. The methods used were essentially the same as described earlier (Parke & Williams, 1953).

Reference compound. In one experiment the diphenylmethyl ester of β-phenyl-D-glucuronide was used. The compound is new and was prepared by treating phenylglucuronide (0·5 g.) dissolved in methanol (5 ml.) with an ethereal solution (10 ml.) of diazodiphenylmethane (0·5 g.) (Elvidge, Linstead, Sims & Orkin, 1950). After standing overnight the crystals which separated were collected and recrystallized from ethanol (yield, 0·6 g.). The *diphenylmethyl ester* formed colourless needles, m.p. 172°, $[\alpha]_D^{20}$ − 72 ± 1° in ethanol (*c*, 1). (Found: C, 68·8; H, 5·8. $C_{25}H_{24}O_7$ requires C, 68·8; H, 5·5%.) (Light absorption in ethanol; $\lambda_{max.}$ 207, 212, 214, 261–262, 267, 274 mμ. with $\epsilon_{max.} \times 10^{-3}$ 17·1, 18·3, 17·1, 1·27, 1·62, 1·08, respectively.)

Experiments with [^{14}C]*benzene*

Phenylglucuronide. Rabbits were fed with [$^{14}C_1$]benzene (cf. Parke & Williams, 1953) and the urine collected for 48 hr. To one-quarter of the urine was added 200 mg. of β-phenyl-D-glucuronide dihydrate (m.p. 162°, cf. Garton, Robinson & Williams, 1949) dissolved in a little water. The glucuronide was then isolated from the urine as the basic lead salt as described by Porteous & Williams (1949). After removal of the lead with H_2S and concentrating, the glucuronide was obtained as a pale-yellow gum. This was purified by dissolving in methanol, filtering and evaporating to dryness *in vacuo*. The residue was recrystallized twice from water and its specific activity determined. The glucuronide (m.p. 161°) was converted into the triacetyl methyl ester (m.p. 115°) and then to phenylglucuronid-

amide (m.p. 225° decomp.) as described by Parke & Williams (1951). The specific activity of each of these derivatives was determined. The glucuronide, ester and amide had the same activity on a molecular basis.

In a second experiment, the isolated glucuronide was dissolved in ethanol and treated with an ethereal solution of diazodiphenylmethane. The phenylglucuronide diphenylmethyl ester was isolated (m.p. and mixed m.p. 170°). The total activity of the glucuronide was unchanged on converting it into this ester. The results are given in Table 1.

Phenylsulphuric acid. Phenol (0·5 g.) was added to half of the urine (see above) which was then made N with respect to HCl. The acidified urine was then heated at 95° for 15 min. to hydrolyse ethereal sulphates. About 5% of the phenylglucuronide present is hydrolysed under these conditions (Garton *et al.* 1949). The mixture was continuously extracted with ether, the ether extract steam distilled and the phenol in the distillate converted into the toluene-*p*-sulphonate (cf. Parke & Williams, 1953) and its specific activity determined. This procedure gives phenol combined as ethereal sulphate after making a small correction of 5% for the phenylglucuronide hydrolysed.

Total phenol. This was estimated after adding 0·5 g. of carrier phenol to one-quarter of the urine by the method described by Parke & Williams (1953). In one experiment catechol and quinol were also determined isotopically (see Table 1). The difference between the total phenol and the phenol obtained by mild acid hydrolysis should be equal to the phenol present as glucuronide determined in the preceding section. Table 1 shows that this is so. Little if any phenylglucuronide could have been derived from other sources such as a hypothetical glucuronide of a 1:2-dihydrobenzene-1:2-diol.

Experiments with [^{14}C]phenol

Preparation of [^{14}C]phenol. The urines of rabbits which had received [$^{14}C_1$]benzene were pooled, made approx. 10N with respect to H_2SO_4 (1 vol. conc. H_2SO_4 to 3 vol. urine) and heated under reflux for 1 hr. The liberated phenols were extracted with ether and then transferred to 2N-NaOH. The alkaline solution was acidified and the phenol recovered by steam distillation. The phenol was converted into the toluene-*p*-sulphonate which was then purified by repeated crystallization from aqueous ethanol. The pure ester (m.p. 96°) was hydrolysed by heating under reflux for several days with 15N-H_2SO_4. The liberated phenol was steam distilled and then extracted from the distillate with ether. The ethereal solution was passed through a short column of alumina, dried over anhydrous Na_2SO_4 and finally evaporated at 0° to yield [^{14}C]phenol as a colourless crystalline solid, m.p. 38°.

Preparation of phenylglucuronide labelled with ^{14}C in the benzene ring. This glucuronide (m.p. 161°) was prepared biologically from the [^{14}C]phenol according to the method of Porteous & Williams (1949).

The recovery of phenylglucuronide from urine. Systematic lead acetate precipitation has long been used as a general method for the isolation of glucuronides from urine, the glucuronides usually precipitating as basic lead salts. ^{14}C-Labelled phenylglucuronide enabled us to test the completeness of this precipitation.

Phenylglucuronide (165 mg.), labelled with ^{14}C, was added to 25 ml. normal rabbit urine. The solution was adjusted to pH 4·5 with 2N-acetic acid and 5 ml. of saturated normal lead acetate added. The precipitate was collected on the centrifuge, dried and its specific activity determined. The filtrate was adjusted to pH 8 with 2N-ammonia and 5 ml. of basic lead acetate solution added (the basic lead acetate solution was made by dissolving 56 g. PbO in a solution of 95 g. of $Pb(OCOCH_3)_2$, $3H_2O$ in 800 ml. water with heating, then cooling and diluting to 1 l.). The basic lead precipitate was collected, washed twice with water, dried and its activity determined. Excessive washing with water was avoided since the progressive removal of electrolyte produced a sol of basic lead salt. The basic lead salt was suspended in 30 ml. water and the lead removed with H_2S.

Table 1. *The excretion of phenylglucuronide and phenylsulphuric acid by rabbits receiving [$^{14}C_1$]benzene orally*

Dose of benzene (mg./kg.)	Isotope dose (μc)	Percentage of dose excreted in 48 hr. as: Total phenol	Phenyl-glucuronide	Phenyl-sulphuric acid*	Catechol	Quinol
150	2·5	21·6	8·1 (7·8†)	13·8	—	—
500	11·0	17·6	6·3 (6·5†)	11·1	—	—
350	9·5	18·2	—	—	4·4	7·5

* Calculated from phenol liberated by mild hydrolysis.
† Calculated from total phenol minus phenylsulphuric acid (i.e. phenol by strong hydrolysis minus phenol by mild hydrolysis).

Table 2. *Recovery of phenylglucuronide from urine by systematic lead-acetate precipitation*

Fraction	Wt. (mg.)	^{14}C concn. (10^{-5} μg./mg.)	Total ^{14}C content (10^{-5} μg.)	Recovery (%)	
Phenylglucuronide added	165	62·0	10 230	—	
Normal lead acetate precipitate	290	1·3	377	4	98
Basic lead acetate precipitate	1505	6·4	9 632	94	
Lead sulphide	880	<0·1	<88	<1	
Glucuronide gum	235	40·0	9 400	92	
Phenylglucuronide diphenylmethyl ester isolated	254	34·5	8 760	85	

The PbS was collected, washed with water and found to contain no radioactivity. The filtrate was concentrated *in vacuo* below 40°. The glucuronide gum thus obtained was purified by dissolving in ethanol, filtering and evaporating to dryness *in vacuo*. The specific activity of the dried material was determined; the glucuronide was then converted into the crystalline diphenylmethyl ester, the activity of which was also determined.

The results are given in Table 2, which shows that 94% of the glucuronide appears in the basic lead acetate precipitate, thus justifying this procedure. Loss of glucuronide in this process obviously occurs in the stages after the precipitation by basic lead acetate.

The metabolism of phenyl acetate. The possibility was considered that phenol might be so rapidly conjugated that it would not readily reach sites in the body where it could be oxidized. An attempt was made to circumvent this by the administration of phenyl acetate. As a criterion of oxidation the formation of catechol was measured.

Two rabbits were given phenyl acetate (350 mg./kg.), one by mouth and the other by intraperitoneal injection. The output of catechol was determined by the method of Azouz, Parke & Williams (1953). The yield of catechol in the urine in 48 hr. was 0·7 and 1·4% of the dose, respectively. Thus phenyl acetate produced essentially the same amount of catechol as did phenol (see Table 3) and is presumably rapidly hydrolysed by esterases to phenol.

Table 3. *The metabolites of orally administered* ^{14}C*-labelled phenol in rabbits*

	Expt. 1	Expt. 2	Expt. 3
Dose of phenol:			
Total (mg.)	160	140	550
(mg./kg.)	60	50	200
Total dose of radioactivity (μc ^{14}C)	0·5	0·5	2
Metabolite excreted (in 2 days after dosing)	As percentage of dose		
Total phenol	95	84	—
Phenylglucuronide*	50	37	—
Phenylsulphuric acid†	45	47	—
Catechol	1·0	0·5	—
Quinol	9·5	10·0	—
Hydroxyquinol	<0·5	—	—
Phenylmercapturic acid	0	—	—
Muconic acid:			
cis-cis-	0	—	0
cis-trans-	—	0	0
trans-trans-	0	0	0
Total urinary activity	105	88	—
Sum of phenols	105·5	94·5	—

* Calculated from the difference between total phenol and phenol liberated by mild hydrolysis.

† Calculated from the phenol liberated by mild hydrolysis.

The metabolism of [^{14}C]*phenol.* The phenol, dissolved in olive oil, was administered orally to rabbits. The urine was collected for 48 hr. The total urinary radioactivity was determined as $BaCO_3$ after combustion of a dried urine sample. Phenol, catechol, quinol, hydroxyquinol, phenylmercapturic acid and *cis-cis-*, *cis-trans-* and *trans-trans-*muconic acids were determined by isotope dilution as described by Parke & Williams (1953). Phenylsulphuric acid and phenylglucuronide were determined as above. The results are given in Table 3.

DISCUSSION

Porteous & Williams (1949) showed that when 0·5 g./kg. of benzene was fed to rabbits, about 11% of the dose was excreted as glucuronides and 10% as ethereal sulphates (total, 21%) of phenol, catechol, quinol and hydroxyquinol. More recently Azouz, Parke & Williams (1952) gave the values 11% glucuronides and 25% ethereal sulphates (total, 36%) which are more in keeping with the value (31%) for total phenols, determined isotopically by Parke & Williams (1953). In the present work we have been able to find out how much of the glucuronide excreted is actually phenylglucuronide and how much of the ethereal sulphate is phenylsulphuric acid (see Table 1), and it appears that most of the former conjugate is phenylglucuronide, whereas only about a half of the latter is phenylsulphuric acid. This confirms the suggestion of Parke & Williams (1951) that the polyphenols of benzene urine are excreted mainly as ethereal sulphates, whereas the glucuronide fraction is almost entirely phenylglucuronide. The ratios of phenylglucuronide to phenylsulphate in both experiments were 0·59 and 0·57. The phenol obtained by acid hydrolysis of benzene urine could arise from phenylsulphuric acid, phenylglucuronide and the hypothetical 1:2-dihydrobenzene-1:2-diol and its glucuronide (cf. naphthalene and anthracene). On gentle hydrolysis the latter compound could give rise to phenylglucuronide (cf. the conversion of 1:2-dihydroanthracene-1:2-diol glucuronide into 1-anthrylglucuronide (Boyland & Levi, 1936)). Table 1 shows that the amount of phenylglucuronide found by direct isolation is almost identical with that calculated from the difference between total phenol and phenol obtained by mild hydrolysis. This suggests that little if any glucuronide of the dihydrodiol of benzene is present in the urine.

Our main interest in the metabolism of phenol was to find out whether it gave rise to *trans-trans-*muconic acid as does benzene. Both phenol and catechol can be oxidized chemically to *cis-cis-*muconic acid by peracetic acid (Böeseken, 1932). However, Table 3 shows that none of the three isomers of this acid is found in the urine of rabbits fed with phenol at levels from 50 to 200 mg./kg., and if any had been formed we could have detected it down to 0·05% of the dose of phenol. From this we can conclude that either phenol is not a precursor of muconic acid *in vivo* or that the conjugation

reactions are too rapid to allow the phenol to reach the site of muconic acid formation. Phenol, however, is appreciably oxidized (10%) to quinol and catechol and if catechol were the precursor of muconic acid, one would have expected the latter to be formed in these experiments. This suggests that neither phenol nor catechol are precursors of muconic acid in the intact animal and we are therefore inclined to the hypothesis that a reduced benzene derivative such as 1:2-dihydrobenzene-1:2-diol may be the precursor of muconic acid. In fact, the formation of this acid might well be an indication of the production of such reduced compounds *in vivo*. Our attempts to synthesize this diol in order to test this hypothesis have, so far, failed.

Most of the phenol fed was excreted in conjugated form and at the level fed (50–60 mg./kg.) the glucuronide and ethereal sulphate were excreted in approximately equal amounts. The values for ethereal sulphate (45 and 47%; see Table 3) confirm the earlier values (36–45%) of Williams (1938) for low levels of phenol (25–50 mg./kg.) (cf. Bray, Thorpe & White, 1952).

SUMMARY

1. Using ^{14}C-labelled benzene it has been shown in rabbits that 6–8% of an oral dose of benzene (150–500 mg./kg.) is excreted as phenylglucuronide and 11–14% as phenylsulphuric acid.

2. [^{14}C]Phenol and [^{14}C]phenylglucuronide have been prepared biosynthetically by feeding rabbits with [^{14}C]benzene and [^{14}C]phenol, respectively.

3. At a dose level of 50–60 mg./kg., orally administered phenol can be almost entirely accounted for as urinary metabolites in 2 days after dosing. These metabolites are phenylglucuronide (40–50%), phenylsulphuric acid (about 45%), quinol (about 10%) and catechol (0·5–1·0%).

4. Orally administered phenol does not give rise to any of the isomers of muconic acid or to phenylmercapturic acid.

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REFERENCES

Azouz, W. M., Parke, D. V. & Williams, R. T. (1952). *Biochem. J.* **50**, 702.

Azouz, W. M., Parke, D. V. & Williams, R. T. (1953). *Biochem. J.* **55**, 130.

Böeseken, J. (1932). *Proc. Acad. Sci. Amst.* **35**, 750.

Boyland, E. & Levi, A. A. (1936). *Biochem. J.* **30**, 728.

Bray, H. G., Thorpe, W. V. & White, K. (1952). *Biochem. J.* **52**, 426.

Elvidge, J. A., Linstead, R. P., Sims, P. & Orkin, B. A. (1950). *J. chem. Soc.* p. 2240.

Garton, G. A., Robinson, D. & Williams, R. T. (1949). *Biochem. J.* **45**, 65.

Parke, D. V. & Williams, R. T. (1951). *Biochem. J.* **48**, 621.

Parke, D. V. & Williams, R. T. (1953). *Biochem. J.* **54**, 231.

Porteous, J. W. & Williams, R. T. (1949). *Biochem. J.* **44**, 46.

Williams, R. T. (1938). *Biochem. J.* **32**, 878.

The Precipitation of 3β-Hydroxysteroids by Digitonin

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Most 3β-hydroxysteroids are precipitated by digitonin in ethanolic or methanolic solution, whilst most 3α-hydroxysteroids and steroids not carrying a hydroxyl group at C-3 are not precipitated by digitonin. This rule, originally suggested by Fernholz (1935), is subject to certain exceptions about which no simple generalizations can be made; a variety of 3β-hydroxysteroids are not precipitated in the usual conditions, and a few other steroids are precipitated. The literature up to 1949 is summarized by Fieser & Fieser (1949) (cf. also Strain,1943). Some further exceptions to the general rule are mentioned in the discussion.

Recent work in this laboratory on the steroids of pregnant mares' urine (Brooks, Klyne, Miller & Paterson, 1952) and on the determination of the '3α' and '3β' ketosteroid fractions of human urine (Haslam & Klyne, 1952), led us to investigate the precipitation of 3β-hydroxysteroids by digitonin in a roughly quantitative manner by the method of Haslewood (1947*a*).

The results show that the configuration at C-5, and the presence or absence of substituents in the *D*-ring area, affect considerably the precipitability of 3β-hydroxysteroids by digitonin. A few miscellaneous experiments with 3β-acetoxysteroids and

Table 1. *Effect of varying concentration of digitonin*

('Solubility product' (S) = [minimum concn. of steroid] × [concn. of digitonin] in moles²/l.².)

Digitonin concn. (before mixing, %)	Minimum final concn. of steroid for pptn. (mg./100 ml.)		S (10^{-8} moles²/l.²)	
	Cholesterol	Dehydro-*epi*androsterone	Cholesterol	Dehydro-*epi*androsterone
1·0	0·2	6·4	3·4	144
0·5	0·4	12·8	3·4	144
0·25	(0·4*) 0·8	25·6	(1·7*) 3·4	144
0·125	(0·8*) 1·6	—	(1·7*) 3·4	—

* Very slight precipitation.

with steroids not carrying a 3β-hydroxyl group are included; it is hoped to carry out a more thorough survey of such compounds later.

EXPERIMENTAL

Materials. Digitonin was used as supplied by British Drug Houses Ltd. or L. Light & Co. Ltd., Wraysbury, Bucks. A 1% (w/v) solution in 90% (v/v) ethanol was kept at 37° until required for use. Most of the steroids used were gifts from colleagues, to whom acknowledgements are made in Tables 2–4.

Method for examination of digitonin precipitability. The steroids were examined using a series of twofold dilutions in absolute ethanol, generally from 128 to 1 mg./100 ml. (sometimes 256 and 512 mg./100 ml. for compounds which did not form precipitates at lower concentrations). Each dilution (1 ml.) was mixed with 1% digitonin solution (4 ml.) in a stoppered tube (giving final steroid concentrations of 25·6 to 0·2 mg./100 ml., rarely 51 and 102 mg./100 ml.) and left to stand for 24 hr. at room temperature (15–20°) (cf. Haslewood, 1947*a* and unpublished data). A blank was set up with each experiment using 1 ml. of ethanol and 4 ml. of 1% digitonin solution; after 24 hr. the blank was usually clear, but occasionally it was slightly cloudy. It was considered that precipitation had occurred if a test sample was more cloudy than the blank. The concentrations given in Tables 2–4 are the minimal concentrations of steroid (in the final reaction mixture) with which precipitation occurred; approximate 'solubility products' may be calculated from these. The sign '>25·6' indicates that 25·6 mg./100 ml. was the highest concentration tested, and that no precipitation occurred.

Effect of varying concentration of digitonin. No attempt was made to carry out a thorough study of the effects of varying conditions, but two common steroids—cholesterol (cholest-5-en-3β-ol) and dehydro*epi*androsterone (3β-hydroxyandrost-5-en-17-one)—were tested with 0·5, 0·25 and 0·125% as well as with 1% digitonin solutions. The results (Table 1) agreed with the expectation that

[minimum concentration of steroid] × [digitonin concentration] = constant.

RESULTS

The results for 3β-hydroxy- and 3β-acetoxy-steroids and for a few miscellaneous compounds are collected in Tables 2–4. The results are expressed as the minimum final concentration of steroid (c, in mg./100 ml.) which gives a precipitate in the conditions of the test.

The data of Table 1 indicate that there is an equilibrium between the digitonin-steroid complex and its components in solution, and it therefore seems permissible to calculate the 'solubility products' (S) of these complexes.

S = [minimum concentration of steroid] × [concentration of digitonin].

Since the term 'solubility product' is generally employed with reference to ionic equilibria, its use for this non-ionic equilibrium will be distinguished by the use of quotation marks. Bergmann & Stein (1939) have used the term 'solubility product' in a similar semi-quantitative fashion for certain amino-acid complexes. Since the tests for digitonin precipitability were done only at a series of twofold dilutions, it would give a false impression of accuracy if values for the 'solubility product' (in moles²/l.²) were calculated using accurate molecular weights. It seems more appropriate to indicate the approximate 'solubility products' (for compounds of molecular weight about 300) corresponding to the various minimal concentrations of steroid (c) (see the head of Table 2).

The results of previous workers (Schönheimer & Dam, 1933; Noller, 1939; Haslewood, 1947*a*), some of which were obtained in slightly different conditions, are given in footnotes to the tables.

Separations of 3β-hydroxysteroids by fractional precipitation with digitonin

The results of the semi-quantitative survey suggested that it might be possible to separate certain mixtures of 3β-hydroxysteroids, e.g. mixtures of 5-epimers, by fractional precipitation with digitonin. Such mixtures are often formed by reduction of Δ^5-unsaturated compounds and, whilst the isolation of the predominant 5α-epimer is easy, the isolation of the 5β-epimer is not (cf. Brooks, Klyne & Miller, 1953, and references there).

Mixtures of two known steroids in ethanol were taken and treated with digitonin in such conditions that the more soluble digitonide would just fail to precipitate, whilst the bulk of the less soluble digitonide would be precipitated.

In a preliminary experiment 3β-hydroxy-5α-(and -5β-) androstan-17-ones were mixed in equal quantities and treated with digitonin. The precipitate (*B* fraction) and the material remaining in solution (*A* fraction) were dried and decomposed in the normal manner with pyridine and ether (Schönheimer & Dam, 1933). The melting points of the recovered steroids showed that, as expected, the 5β-isomer (m.p., 153–155°; less easily precipitable) was concentrated in the *A* fraction (m.p. 135–140°), whilst the 5α-isomer (m.p., 174–175°; more easily precipitated) was concentrated in the *B* fraction (m.p. 162–170°; after recrystallization from ethyl acetate:light petroleum, m.p., 173–175°).

Table 2. *Digitonin precipitability of 3β-hydroxysteroids*

(Minimum concentration for precipitation (*c*) is expressed as mg. steroid/100 ml. of solution after mixing with digitonin. '?' indicates very slight precipitation. References are given only for less common compounds.)

Approximate values of 'solubility products' (*S*) corresponding to given minimum concentrations of steroid for precipitation (*c*). Mol.wt. assumed 300.

c (mg./100 ml.)	$10^8 \times S$ (moles^2/l.^{2})	*c* (mg./100 ml.)	$10^8 \times S$ (moles^2/l.^{2})
0·2	4	6·4	140
0·4	8	12·8	280
0·8	16	25·6	550
1·6	35	51	1100
3·2	70	102	2200

Compound	Minimum concn. for pptn. (*c*) (mg./100 ml.)	Sources and references
Monohydroxy compounds		
5α-Androstan-3β-ol	0·4	*a*
Androst-5-en-3β-ol	1·6	*a*
5α-Cholestan-3β-ol	0·2*†	—
Cholest-5-en-3β-ol	0·2*†	—
5α-Pregnan-3β-ol	0·4	*b*
Pregn-5-en-3β-ol	0·4	*b*
Stigmasta-5:22-dien-3β-ol	0·2	—
5α-Ergost-7-en-3β-ol	0·2	*b*
5α-Ergosta-7:22-dien-3β-ol	0·2	*b*
5α-Ergost-8(14)-en-3β-ol	0·8	*b*
5α-Ergost-14-en-3β-ol	0·2	*b*
3-Methyl-5α-androstan-3β-ol	1·6	*c*, *l*
5β-Cholestan-3β-ol (coprostanol)	3·2*†	*d*
Hydroxyacids and hydroxyesters		
3β-Hydroxychol-5-enic acid	0·8	*e*
Methyl 3β-hydroxychol-5-enate	0·4	*e*
Methyl 3β-hydroxy-5α-cholanate	0·4	*b*
Methyl 3β-hydroxy-5α-etianate	0·4	*b*
Methyl 3β-hydroxy-eti-5-enate	0·4	*b*
Hydroxyketones		
3β-Hydroxy-5α-cholestan-6-one	0·4	—
3β-Hydroxy-5α-cholestan-7-one	0·4	—
3β-Hydroxycholest-5-en-7-one	0·4	—
3β-Hydroxy-5α-androstan-7-one	0·8	*a*, *m*
3β-Hydroxy-5α-androstan-17-one	6·4	*f*
3β-Hydroxyandrost-5-en-17-one	6·4	*f*
3β-Hydroxy-5α-pregnan-20-one	0·2	*g*
3β-Hydroxypregn-5-en-20-one	0·8	*e*
3β-Hydroxy-5α-*D*-homoandrostan-17*a*-one	1·6	—
3β-Hydroxy-*D*-homoandrost-5-en-17*a*-one	3·2	*h*, *n*
3β-Hydroxy-5α-des-*D*-androstan-14-one	26	*i*, *o*
3β-Hydroxy-des-*D*-androst-5-en-14-one	26	*i*, *p*
3β-Hydroxy-5β-androstan-17-one	>51?	*i*
3β-Hydroxy-5β-pregnan-20-one	51	*j*
Sapogenins		
5α:22*a*-Spirostan-3β-ol (tigogenin)	3·2‡	*j*
22*a*-Spirost-5-en-3β-ol (diosgenin)	3·2	*j*
5β:22*b*-Spirostan-3β-ol (sarsasapogenin)	12·8‡	*j*

Table 2 (*cont.*)

Compound	Minimum concn. for pptn. (*c*) (mg./100 ml.)	Sources and references
Dihydroxy compounds		
Cholest-5-ene-3β:4β-diol	3·2	—
5α-Androstane-3β:17β-diol	26	*f*
Androst-5-ene-3β:17α-diol	26	*i*
Androst-5-ene-3β:17β-diol	51	*i*
5α:14β-Androstane-3β:17α-diol	26	*a*, *q*
5α-Pregnane-3β:20α-diol	0·8	—
5α-Pregnane-3β:20β-diol	6·4	—
Pregn-5-ene-3β:20α-diol	3·2	—
Pregn-5-ene-3β:20β-diol	12·8	—
5α-Pregn-16-ene-3β:20β-diol	1·6	*j*, *r*
Pregna-5:16-diene-3β:20α-diol	3·2	*k*, *s*
Pregna-5:16-diene-3β:20β-diol	3·2	*j*, *r*, *s*
5α-*D*-Homoandrostane-3β:17aβ-diol	3·2	*h*
17α-Methyl-5α-*D*-homoandrostane-3β:17aβ-diol (uranediol)	26	—
17α-Ethynylandrost-5-ene-3β:17β-diol	>51	*i*
5β-Pregnane-3β:20β-diol	>51	*j*
Dihydroxyketones		
3β:14α-Dihydroxy-5α-androstan-17-one	>26	*a*, *q*
3β:14α-Dihydroxyandrost-5-en-17-one	>26	*a*, *q*
3β:17α-Dihydroxy-5α-pregnan-20-one	0·4	*j*

* Haslewood (1947*a*) found that these three compounds were precipitated at minimum concentrations of $<0{\cdot}2$, $<0{\cdot}2$ and 6 mg./100 ml. respectively.

† Schönheimer & Dam (1933) gave values for the solubilities of the digitonides of these compounds (in 96%, v/v, ethanol) from which the following solubility products are calculated: $10^8 \times S = 0{\cdot}9$, 1·5, 18 moles²/l.².

‡ Noller (1939) gave the solubility products S (in moles²/l.²) for these compounds as 16×10^{-8} and 970×10^{-8} respectively.

Sources (in some cases the compounds used were prepared from starting materials provided by the following workers): *a*, Dr H. B. MacPhillamy, CIBA Pharmaceuticals Inc., Summit, N.J.; *b*, Dr D. H. R. Barton, Birkbeck College, London; *c*, Prof. V. Prelog, Eidg. Technische Hochschule, Zürich; *d*, Prof. G. A. D. Haslewood, Guy's Hospital Medical School, London; *e*, Dr W. L. C. Veer, N. V. Organon, Oss, Netherlands; *f*, Dr V. Petrow, British Drug Houses Ltd., London; *g*, Dr J. A. Moore, Parke Davis and Co., Detroit, Michigan; *h*, Dr H. Heusser, Eidg. Technische Hochschule, Zürich; *i*, Dr A. Wettstein, CIBA A-G., Basel; *j*, Dr C. Djerassi and Dr G. Rosenkranz, Syntex S.A., Mexico City, and Wayne University, Detroit, Michigan; *k*, Prof. A. Ercoli, Laboratori Ricerche Vister, Casatenovo Brianza, Como, Italy.

References: *l*, Ruzicka, Meister & Prelog (1947); *m*, Heard & McKay (1946); *n*, Heusser, Herzig, Fürst & Plattner (1950); *o*, Achtermann (1934); *p*, Köster & Logemann (1940); *q*, St André, MacPhillamy, Nelson, Shabica & Scholz (1952); *r*, Marker *et al.* (1941); *s*, Ercoli & de Ruggieri (1952); *t*, Butenandt & Schmidt-Thomé (1939).

Table 3. *Digitonin precipitability of 3β-acetoxysteroids*

(Concentrations and references as in Table 2.)

Compound	Minimum concn. for pptn. (*c*) (mg./100 ml.)	Sources
3β-Acetoxy derivative of		
5α-Cholestane	51	—
Cholest-5-ene	>51	—
5α-Pregnane	51	*b*
Methyl 5α-cholanate	>26	*b*
Androst-5-en-17-one	>51	*f*
5α-Androstan-17-one	>102	*f*
5α-*D*-Homoandrostan-17*a*-one	>51	—
5α-Pregnan-20-one	26	*g*
Pregn-5-en-20-one	26	*e*
5α-Pregn-16-en-20-one	26	*j*
5β-Pregnan-20-one	>51	*j*

Table 4. *Digitonin precipitability; miscellaneous compounds*

(Concentrations and references as in Table 2.)

	Minimum concn. for pptn. (*c*) (mg./100 ml.)	Sources and references
5α-Cholestan-3α-ol	102*	*b*
5β-Cholestan-3α-ol	>102	*b*
3β-Methyl-5α-androstan-3α-ol	51	*c*, *l*
3α-Hydroxy-5α-androstan-17-one	>102	*e*
5α-Cholestan-2α-ol	>102	*c*, *u*
5α-Cholestan-2β-ol	51	*c*, *u*
5α-*A*-Norandrostan-2α-ol	102	*c*, *v*
5α-*A*-Norandrostan-2β-ol	12·8	*c*, *v*
5α-Cholestan-3-one	6·4†	—
Cholest-4-en-3-one	>102†	—

* Noller (1939) found the solubility product of this compound to be 940×10^{-8} moles²/l.².

† Haslewood (1947*a*) found that these two compounds were precipitated at minimum concentrations of 60 and 350 mg./100 ml. respectively.

References: *u*, Ruzicka, Plattner & Furrer (1944); *v*, Ruzicka, Prelog & Meister (1945).

Table 5. *Separation of 3β-hydroxysteroids with digitonin*

(In the androstane series the two compounds were dissolved in ethanol (35 ml.) and treated with digitonin (1·40 g.) in 90% (v/v) ethanol (140 ml.). After standing 24 hr. at room temperature the precipitate (*B*-fraction) was centrifuged and washed once with its own volume of 92% ethanol, the washings being added to the supernatant (*A* fraction). Similar proportions were used in the pregnane series.)

	Androstane series (3β-Hydroxy-17-ketones)		Pregnane series (3β-Hydroxy-20-ketones)	
Mixture taken	5β-Compound	Δ⁵-Compound	5β-Compound	Δ⁵-Compound
Wt. (mg.)	70	70	43	43
M.p. (°)	153–155	152–154	143–144	191–194
$[\alpha]_D$ in EtOH	+89°	+10°	+101°	+25°
Fractions separated	*A*	*B*	*A*	*B*
Wt. (mg.)	56	84	36	48
M.p. (°)	153–156	105–135	134–138	165–183
Content of Δ⁵-compound (%)				
By Allen† reaction	10	72	—	—
By optical rotation	13	82	26	88
Product after recrystallization				
M.p.	152–154	134–140*	141–142	185–189

* This compound sometimes crystallizes in needles, m.p. 140–141°.
† Allen, Hayward & Pinto (1950).

No simple method exists for determining the proportions of 5α- and 5β-isomers in a mixture; further experiments were therefore carried out with mixtures of 5β-compounds (less precipitable) and the related Δ⁵-unsaturated compounds (more precipitable), since the proportions of unsaturated material in the two fractions can be determined by optical-rotation measurements and, for 17-ketones of the androstane series, by the sulphuric acid colour reaction of Allen, Hayward & Pinto (1950).

The results (Table 5) show that the less precipitable (5β) compounds can easily be obtained pure from the *A* fractions after one crystallization. The more precipitable (Δ⁵) compounds are largely contained in the *B* fraction, but are contaminated with 5β-compound even after recrystallization.

DISCUSSION

3β-*Hydroxysteroids*

5α- and 5β-Epimers. The most important point which emerges from this work is the fact that 3β-hydroxysteroids of the 5β-series are precipitated by digitonin only at much higher concentrations than those which are minimal for the corresponding compounds of the 5α-series. The differences are such that digitonin precipitation in controlled conditions may be used to separate 5α- and 5β-isomers. Such isomers are difficult to separate by chromatography.

Haslewood (1947*b*) found that whilst 5α-cholestan-3-one (cholestanone), cholest-4-en-3-one and cholest-5-en-3-one in high concentrations were precipitated by digitonin, 5β-cholestan-3-one (coprostanone) was not. He suggested that *cis*-union of rings *A* and *B* might be less favourable to digitonide formation than the flat configuration in *trans*-, Δ⁴-, or Δ⁵-compounds. This seems a reasonable hypothesis, especially in view of what is now known regarding the preferred conformations of 5β (*A*/*B cis*) and 5α (*A*/*B trans*) steroids (Barton, 1950, 1953). The present experimental work supports this suggestion.

Olefinic linkages. Olefinic linkages at C-5, C-7, C-8 or C-14 usually make no appreciable difference to the digitonin precipitability of 3β-hydroxy compounds. Androst-5-en-3β-ol and some of the pregn-5-ene-3:20-diols are exceptions. Hirschmann (1943) found that whilst 5α-androstane-3β:16α:17β-triol was readily precipitated with digitonin, the corresponding Δ⁵-compound was only partially precipitated.

Side chains. Different hydrocarbon, acid or ester side chains at C-17, in the normal 17β-configuration, usually make no appreciable difference to the digitonin precipitability. Fieser & Fieser (1949) give references to a number of compounds carrying 17α side chains which are not precipitable. The spiroketal side chain of the sapogenins reduces the digitonin precipitability greatly.

'Lanosterol' (lanostadienol), which is now known to be 4:4:14-trimethylcholesta-8(9):24-dien-3β-ol (Voser, Mijović, Heusser, Jeger & Ruzicka, 1952) gives a sparingly soluble digitonide (Ruzicka, Denss & Jeger, 1945). 'Dihydrolanosterol' (lanostenol), in which the 24:25 double bond is saturated, gives a digitonide which is relatively easily soluble.

Keto groups. Ketone groups at C-6, C-7 and C-20 make little or no difference to the digitonin precipitability (except in the case of 5β-pregnane-3β-

ol-20-one, which is much less easily precipitable than 5β-cholestan-3β-ol. Ketone groups at C-17 (and at C-17*a* in the *D*-homosteroid series) reduce the digitonin precipitability considerably. The two des-*D*-14-ketones were precipitated only at high concentrations. (These are steroids lacking ring *D*; for nomenclature see *J. chem. Soc.*, 1952, p. 5060.)

Additional hydroxyl groups in positions other than C-3. Hydroxyl groups at C-20 reduce the digitonin precipitability somewhat (20β-hydroxyl more than 20α-), and hydroxyl groups at C-17 reduce it even more so. Butenandt, Tscherning & Hanisch (1935) found that 5α-androstane-3β:17β-diol was not precipitable by digitonin. Hirschmann & Hirschmann (1950) stated that the corresponding Δ^5-compound gave a rather soluble digitonide. (Both these compounds were called 17α by the original authors.) The pregnanetriol of pregnant mares' urine (5α-pregnane-3β:16α:20β-triol; Haslewood, Marrian & Smith, 1934) and some related compounds (Hirschmann, Hirschmann & Daus, 1949) are not precipitated by digitonin.

Marker, Rohrmann & Wittle (1938) isolated uranediol (17α-methyl-*D*-homoandrostane-3β:17*a*β-diol) from pregnant mares' urine by a method involving fractional precipitation with digitonin. Brooks *et al.* (1952), using the same method, obtained uranediol, 5α-pregnane-3β:20β-diol, and 5α-pregnane-3β:20α-diol from the '$\beta 2$' fraction (more soluble digitonides).

3β-Acetoxyl compounds

3β-Acetoxy-5α-pregnan-20-one is precipitable by digitonin (Butenandt & Mamoli, 1935), although it has no free 3β-hydroxyl group. This fact was used by Butenandt and Mamoli and by Shoppee (1949) in separating this compound from its 17α-isomer. Whitman & Schwenk (1940) claimed that precipitation of 3β-acetoxypregn-5-en-20-one by digitonin could be used to separate this compound from other steroid acetates.

We have found that some other 3β-acetoxyl compounds are precipitated at high concentrations (Table 3).

Miscellaneous compounds

Cholestan-2β-ol (Ruzicka, Plattner & Furrer, 1944; configuration proved by Fürst & Plattner, 1949) was precipitated at high concentration; its 2α-epimer could not be precipitated at the highest concentration tested. The *A*-norandrostan-2'β'-ol (Ruzicka, Prelog & Meister, 1945) and 3'α'-methyl-androstan-3'β'-ol (Ruzicka, Meister & Prelog, 1947), configurations of which were allotted on the basis of their digitonin precipitability, were precipitated at concentrations appreciably lower than their 2'α'- and 3'α'-hydroxy-epimers.

Some recent examples of compounds other than 3β-hydroxysteroids which are precipitated by digitonin are as follows: 5α-pregn-2-en-20-one (Lieberman, Dobriner, Hill, Fieser & Rhoads, 1948); 3β-aminocholestane (Dodgson & Haworth, 1952) (the amino group almost certainly has the β (equatorial) configuration, since the compound was obtained by reducing an oxime with sodium and amyl alcohol (cf. Barton, 1953)); conessine (Bertho, 1950), shown by Haworth, McKenna & Whitfield (1953) to be a 3β-dimethylaminopregn-5-ene derivative.

SUMMARY

1. The precipitation of 3β-hydroxysteroids by digitonin has been studied in a roughly quantitative manner by the method of Haslewood (1947*a*).

2. 3β-Hydroxysteroids of the 5α-(*A*/*B*-*trans*) series and of the Δ^5-unsaturated series are precipitated by digitonin in much higher dilution than are the corresponding steroids of the 5β-(*A*/*B*-*cis*) series. This difference in behaviour with digitonin can be used in the separation of such steroids.

3. The behaviour of some 3β-acetoxysteroids and other compounds with digitonin has been studied.

We are most grateful to the many colleagues named in Table 2 who generously provided steroids for this work. We are indebted to Prof. G. A. D. Haslewood for some unpublished data.

REFERENCES

Achtermann, T. (1934). *Hoppe-Seyl. Z.* **225**, 141.
Allen, W. M., Hayward, S. J. & Pinto, A. (1950). *J. clin. Endocrin.* **10**, 54.
Barton, D. H. R. (1950). *Experientia*, **6**, 316.
Barton, D. H. R. (1953). *J. chem. Soc.* p. 1027.
Bergmann, M. & Stein, W. H. (1939). *J. biol. Chem.* **128**, 217.
Bertho, A. (1950). *Liebigs Ann.* **569**, 1.
Brooks, R. V., Klyne, W. & Miller, E. (1953). *Biochem. J.* **54**, 212.
Brooks, R. V., Klyne, W., Miller, E. & Paterson, J. Y. F. (1952). *Biochem. J.* **51**, 694.
Butenandt, A. & Mamoli, L. (1935). *Ber. dtsch. chem. Ges.* **68**, 1847.
Butenandt, A. & Schmidt-Thomé, J. (1939). *Ber. dtsch. chem. Ges.* **72**, 1690.
Butenandt, A., Tscherning, K. & Hanisch, G. (1935). *Ber. dtsch. chem. Ges.* **68**, 2097.
Dodgson, D. P. & Haworth, R. D. (1952). *J. chem. Soc.* p. 67.
Ercoli, A. & de Ruggieri, P. (1952). *Farmaco*, **7**, 11.
Fernholz, E. (1935). *Hoppe-Seyl. Z.* **232**, 97.
Fieser, L. F. & Fieser, M. (1949). *Natural Products Related to Phenanthrene*, 3rd ed. p. 648. New York: Reinhold.

Fürst, A. & Plattner, P. A. (1949). *Helv. chim. acta*, **32**, 275.
Haslam, R. M. & Klyne, W. (1952). *Lancet*, **1**, 285.
Haslewood, G. A. D. (1947*a*). *Biochem. J.* **41**, xli.
Haslewood, G. A. D. (1947*b*). *Biochem. J.* **41**, 639.
Haslewood, G. A. D. Marrian, G. F. & Smith, E. R. (1934). *Biochem. J.* **28**, 1316.
Haworth, R. D., McKenna, J. & Whitfield, G. H. (1953). *J. chem. Soc.* p. 1102.
Heard, R. D. H. & McKay, A. F. (1946). *J. biol. Chem.* **165**, 677.
Heusser, H., Herzig, P. T., Fürst, A. & Plattner, P. A. (1950). *Helv. chim. acta*, **33**, 1093.
Hirschmann, H. (1943). *J. biol. Chem.* **150**, 363.
Hirschmann, H. & Hirschmann, F. B. (1950). *J. biol. Chem.* **187**, 137.
Hirschmann, H., Hirschmann, F. B. & Daus, M. A. (1949). *J. biol. Chem.* **178**, 751.
Köster, H. & Logemann, W. (1940). *Ber. dtsch. chem. Ges.* **73**, 298.
Lieberman, S., Dobriner, K., Hill, B. R., Fieser, L. F. & Rhoads, C. P. (1948). *J. biol. Chem.* **172**, 263.
Marker, R. E., Rohrmann, E. & Wittle, E. L. (1938). *J. Amer. chem. Soc.* **60**, 1561.
Marker, R. E., Turner, D. L., Wagner, R. B., Ulshafer, P. R., Crooks, H. M. & Wittle, E. L. (1941). *J. Amer. chem. Soc.* **63**, 779.
Noller, C. R. (1939). *J. Amer. chem. Soc.* **61**, 2717.
Ruzicka, L., Denss, R. & Jeger, O. (1945). *Helv. chim. acta*, **28**, 759.
Ruzicka, L., Meister, P. & Prelog, V. (1947). *Helv. chim. acta*, **30**, 867.
Ruzicka, L., Plattner, P. A. & Furrer, M. (1944). *Helv. chim. acta*, **27**, 524.
Ruzicka, L., Prelog, V. & Meister, P. (1945). *Helv. chim. acta*, **28**, 1651.
St André, A. F., MacPhillamy, H. B., Nelson, J. A., Shabica, A. C. & Scholz, C. R. (1952). *J. Amer. chem. Soc.* **74**, 5506.
Schönheimer, R. & Dam, H. (1933). *Hoppe-Seyl. Z.* **215**, 59.
Shoppee, C. W. (1949). *J. chem. Soc.* p. 1671.
Strain, W. H. (1943). In *Organic Chemistry*, ed. by Gilman, H. 2nd ed., vol. 2, p. 1341. New York: Wiley.
Voser, W., Mijović, M. V., Heusser, H. Jeger, O. & Ruzicka, L. (1952). *Helv. chim. acta*, **35**, 2414.
Whitman, B. & Schwenk, E. (1940). U.S. patent. 2, 221, 826; *Chem. Abstr.* (1941), **35**, 1412.

The Lysing Action of Enzymes on a Sample of Mycelium of *Penicillium griseofulvum* Dierckx

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Soil organic matter is composed, at least in part, of living micro-organisms and those parts of their remains which are resistant to chemical and enzymic decomposition. A knowledge of the action of enzymes on fungal mycelium may perhaps give some idea of the composition of this resistant fraction. An examination has, therefore, been made of the breakdown by enzymic action of a sample of mycelium of *Penicillium griseofulvum* Dierckx, the composition of which has been studied by Smithies (1952).

The effect of autolysis on the composition of mould mycelium and the nature of the products liberated have been investigated (e.g. by Woolley & Peterson, 1937) usually with special regard to the protein constituent and the production of amino acids, or the yield of a particular metabolic product.

An interesting point is mentioned by Foster (1949) that, whereas in certain bacteria and actinomycetes the phenomenon of self digestion is well known, this does not occur amongst the filamentous fungi, and there remains an enzyme-resistant portion.

This paper deals with the liberation and breakdown of the carbohydrate, protein and chitin constituents of the mycelium, both on autolysis of the air-dried mycelium and on treatment with enzymes from other sources.

MATERIALS AND GENERAL METHODS

Unless otherwise stated, these were as given by Smithies (1952).

Protein. This was estimated by hydrolysis of mycelium with 6N-HCl and determination of α-amino-acid N by the ninhydrin-CO_2 titration method of Van Slyke, MacFadyen & Hamilton (1941), which represents about 72% of the total protein N (Smithies, 1952).

Chitin. This was estimated in mycelium samples by determination of glucosamine hydrochloride in 6N-HCl hydrolysates (Smithies, 1952).

Carbohydrate. This was determined in mycelium by first taking up the sample in 72% (w/w) H_2SO_4 and applying the orcinol method of Pirie (1936) as used by Smithies (1952).

Snail enzyme. This was obtained from the crops and guts of starved snails (*Helix aspersa*) as described by Bawden & Pirie (1946).

Trypsin. Suspensions of dry preparations from pancreas were used as described by Holden & Tracey (1950).

Papain. This was prepared and activated as by Tracey (1948).

Enzyme 19 *AP* (*Rohm and Haas Co., Philadelphia*), *preparations from* Aspergillus aureus *and* Penicillium expansum. These were prepared as by Holden (1950).

Extraction of material soluble in organic solvents. The sample (0·2–0·5 g.) was suspended in a small filter-paper bag (acid-resistant Whatman no. 50), over refluxing ethanol (25 ml. containing two drops of 10 N-HCl) for about 16 hr. and the extract, dried at 100–105°, weighed. This method gave results similar to those obtained when larger samples of mycelium were extracted with organic solvents before and after a mild acid hydrolysis, and the fat extracts combined.

N-Acetylglucosamine. This was estimated in mycelium autolysates by the method of Morgan & Elson (1934) and also by a modification of this method, whereby interference due to the presence of α-amino acids and sugars is removed by a preliminary acetylation (Smithies, 1953).

Manometric measurements. These were made in the Warburg apparatus at 25°. The volume of the reaction mixtures was 3 ml. O_2 uptakes were measured directly, with KOH in the centre cups.

Incubations. These were in general carried out at 37° in stoppered centrifuge tubes, with a drop of toluene added to maintain sterility.

EXPERIMENTS AND RESULTS

Autolysis of mycelium

These experiments were made on a batch of mycelium grown in November 1949. A portion, referred to as 'fresh mycelium', was autolysed only 7 days after harvesting during which time it was allowed to dry in air at room temperature. Further autolysis experiments on the same batch were carried out after a lapse of 2½–3 yr. This portion of the sample was powdered in a small laboratory mill and stored at room temperature in a stoppered glass jar and is subsequently referred to as 'stored mycelium'.

Autolysis of 'fresh mycelium'. The sample (50 g.) was macerated in a blender and the aqueous suspension, containing thymol crystals, made up to 500 ml. and kept at room temperature in a flask loosely plugged with cotton wool. The pH (initially 5·5) gradually increased to pH 8·0.

After 15 days a sample was removed for extraction of the residual suspended mycelium with 1% (w/v) NaOH following the procedure of Bohonos, Woolley & Peterson (1942). The extract was acidified with glacial acetic acid, and the precipitate (10 mg./g. mycelium) which formed at pH 3 collected. After purification it had the approximate composition (moisture free): N, 11·4; protein, 65; carbohydrate (as hexosan), 10; chitin, 2%. Bohonos *et al.* claimed that their protein preparation (from *A. fischeri*) was resistant to digestion by proteases.

The rest of the autolysate remained at room temperature for about 3 months, without any contamination by micro-organisms being observed. After this it was corked and stored at 0–2°. Samples were withdrawn when required.

The final chocolate-brown autolysate had a dry-matter content which accounted for only about 65% of the original solid material, the remainder having been lost as volatile products of autolysis. The composition of the autolysate and the recovery of the various components is given in Table 1. No free α-amino acids, *N*-acetylglucosamine or reducing sugar could be detected. The solution had a small reducing value, but that this was not due to sugar was shown by the fact that the whole of the carbohydrate was colloidal, and no sugar could be detected by paper chromatography.

Autolysis of 'stored mycelium'. The results of a typical experiment are contained in Table 2. Very little change takes place in the composition of the mycelium on storage. Analyses performed within the first 6 months after harvesting and after 3 yr. storage, showed that extracts of the mycelium made at 100° contained, in each case, only a trace of reducing sugar and *N*-acetylglucosamine. Ammonia accounted for 2% of the total N at both times, but the amount of free α-amino-acid N increased from about 3 to 7% of the total N. Slow hydrolysis of protein therefore occurs on storage.

Table 3 compares amounts of mycelial components dispersed by autolysis and by water at 100°, and gives some idea of the increase in extraction due to enzyme action.

Table 1. *Effect of autolysis for 3 months on the composition of mycelium which had been air-dried for 7 days only, after harvesting*

	Composition		Percentage recovery of component in autolysate (total recovery of dry matter =65%)	Composition		Percentage of total component in autolysate found in suspended material
	Of original mycelium	Of autolysate (M_A)		Dispersed or dissolved material in autolysate (30% of total dry matter)	Suspended material in autolysate (70% of total dry matter) (M_B)	
N (mg./g. dry wt.)	39	47	78	118	19	28
P (mg./g. dry wt.)	7·2	11·8	107	31	4	24
Material soluble in organic solvents (mg./g. dry wt.)	330	270	53	540	180	46
Total carbohydrate (as hexosan) (mg./g. dry wt.)	390	480	80	320	540	80
Protein (mg./g. dry wt.)	150	90	39	200	50	39
Protein N (% of total N)	63	32	—	34	43	—
Chitin (mg./g. dry wt.)	60	50	56	23	68	89
Chitin N (% of total N)	11	7	—	1·4	25	—
NH_3-N (% of total N)	2	36	—	42	0	—

Table 2. *The effect of autolysis on the composition of stored mycelium*

(2·0 g. mycelium, containing 1·8 g. dry matter, incubated in 20 ml. water at 37° for 20 days. After centrifuging, the supernatant was combined with one water wash of the undispersed residue, which was air dried and powdered.)

	Composition			Percentage of total component of mycelium found in suspended material
	Stored mycelium	Suspended material in autolysate M_C (65% recovery of dry matter)	Dispersed material in autolysate (33% recovery of dry matter)	
N (mg./g. dry wt.)	39	25	65	42
P (mg./g. dry wt.)	7·2	3	17	27
Material soluble in organic solvents (mg./g. dry wt.)	330	220	Not estimated	43
Total carbohydrate (as hexosan) (mg./g. dry wt.)	390	425	390	71
Reducing sugar (as glucose) (mg./g. dry wt.)	Trace	0	180	—
Protein (mg./g. dry wt.)	150	90	Not estimated	39
Protein N (% of total N)	52	60	—	—
Free α-amino-acid N (% of total N)	7	0	45	—
Ammonia N (% of total N)	2	0	10	—
Chitin (mg./g. dry wt.)	60	65	Not estimated	70
Chitin N (% of total N)	11	18	—	—
N-Acetylglucosamine N (% of total N)	0·2		1·6	—

Table 3. *Increase in extraction of mycelium due to enzyme action*

	Percentage of constituent of stored mycelium dispersed	
	On autolysis	On extraction with water at 100°
Total dry matter	34	24
Nitrogen	55	37
Carbohydrate	29	12
Protein	61	15
Chitin	30	Trace only

Enzymic breakdown of mycelium constituents

Carbohydrate. The fresh wet mycelium, immediately after harvesting, had a noticeable cellulase activity, sufficient to make it difficult to dialyse extracts using cellophan membranes at room temperature. The effect was no longer apparent after air drying, and, perhaps because of this, the carbohydrate of the fresh air-dried mycelium is the most stable of the constituents analysed (Table 1). The autolysate contained no reducing sugar and the oxidases bringing about its destruction were still weakly active in the stored mycelium, which took up a small amount of O_2 when shaken in the Warburg apparatus in 0·1 M-sodium phosphate buffer (pH 7, sat. with thymol). The amount taken up was increased in the presence of glucose, but ethanolamine or DL-alanine caused no such change, showing the absence of amine oxidase or amino-acid oxidase. In a typical experiment 300 mg. of mycelium in 2 hr. took up 45 μl. O_2. In the presence of 0·2 ml. 0·1 M-glucose this was increased to 63 μl. Complete oxidation of this amount of glucose to gluconic acid requires 440 μl. O_2. After incubation of the stored mycelium 70% of the total carbohydrate remained undispersed and the 14% obtained as reducing sugar in solution was identified as glucose by paper chromatography. Glucose only was recognized by this means in acid hydrolysates of the carbohydrate of M_A and M_C (Tables 1 and 2). Glucose, together with traces of galactose and mannose, was detected in acid hydrolysates of stored mycelium.

The breakdown was studied of the carbohydrate (6–8 mg./sample) of M_B and M_C by various enzymes in 0·1 M-sodium acetate buffer (pH 5) at 37°. In preliminary experiments, the hydrolysates (total vol. 2 ml.) were incubated 48 hr. and, after centrifuging, the total carbohydrate and reducing sugar in the supernatant determined. The following preparations dispersed less than 10% of the total carbohydrate (allowing for blanks carried out on the enzyme alone), and no further work was done using them: enzyme 19 *AP* (0·5 ml.); preparations from *A. aureus* and *P. expansum* (1 ml.); trypsin activated with NaCl (0·2 ml. at pH 7). The snail enzyme (0·5 ml.) liberated about 35% of the carbohydrate of M_B and 50% of M_C (Tables 1 and 2). In either case no further carbohydrate was dispersed after five such incubations, and the total amounts liberated were: M_B, 60% of the total carbohydrate and 108 mg. reducing sugar (as glucose)/100 mg. total dispersed carbohydrate; M_C, 85% of the total carbohydrate and 88 mg. reducing sugar (as glucose)/100 mg. total dispersed carbohydrate.

Glucose was the only sugar detected in the digests and the high values of the reducing sugar (of which less than 10% is due to *N*-acetylglucosamine liberated by the chitinase activity of the juice) indicate that no oligosaccharides accumulate.

The mycelial carbohydrate is not cellulose, but the efficiency of snail juice, compared with other enzyme sources, in digesting it, parallels its efficiency in breaking down cellulose.

Protein. The mycelium contains strong protease activity, which persists on storage. In the fresh mycelium, however, amino-acid oxidase is also present, amino acids do not accumulate on autolysis and the ammonia formed constitutes 36% of the total N, although, since the pH rises from 5·5 to 8, some is lost. The oxidase is quite inactive in the stored mycelium, amino acids produced by the stable protease accumulate in solution, and there is practically no change in pH.

The proteolytic enzyme was studied by incubating 40–60 mg. stored mycelium in 1 ml. water or buffer solution at 37° for 24 hr. under specified conditions. The amount of proteolysis was estimated by comparing the amount of α-amino-acid N found, with the total which would be produced on acid hydrolysis.

In sodium diethylbarbiturate-sodium acetate buffer (Michaelis, 1931), it had an optimum activity at pH 7·5–8·0; at pH 5 it was 80%, and at pH 3·5, 70% as active. The protease, in this resemblance in optimum pH to the trypsin activity of pancreas extracts, is similar to other mould proteases that have been studied (Benger, Johnson & Peterson, 1937; Dion, 1950). At the optimum pH, up to 40% of the protein was hydrolysed after 24 hr., and a maximum of 70% after a week's incubation.

The resistance of various mycelium preparations to digestion by trypsin was studied, trypsin being chosen because of its resemblance to fungal protease and the fact that Bohonos *et al.* (1942) found it to be the most effective of the enzymes tried by them in the attempted digestion of a resistant protein extract of mycelium. Furthermore, preliminary experiments on *P. griseofulvum* protein showed trypsin to be about twice as effective as activated papain. The results are given in Table 4. The protein of autolysed mycelium is more resistant than that of the original stored mycelium, although there is no significant difference between the various autolysis preparations. In particular, the dispersed protein of the fresh-mycelium autolysate, and also the alkali-extracted protein of the stored mycelium, might have been expected to show enhanced resistance to autolysis.

Table 4. *Digestion of mycelium protein with trypsin*

(The sample, containing about 0·4–0·6 mg. protein N + 1 ml. 0·1 M-sodium phosphate buffer (pH 7·5) + 0·2 ml. 1% (w/v) trypsin, incubated 24 hr. at 37°.)

Preparation	α-Amino-acid N as % of total found on acid hydrolysis
Gelatin	36
*Stored mycelium	38–43
M_A†	17
M_B†	18–20
M_C‡	24
Alkali-extracted protein	22–25
Dispersed protein in fresh-mycelium autolysate	23

* These samples contained 0·2–1·0 mg. protein N. The mould protease was deactivated by boiling.
† Table 1.
‡ Table 2.

A mycelium sample (0·4 g.) was subjected to autolysis in 5 ml. 0·1 M sodium phosphate buffer (pH 7·5) for 7 days, followed by three incubations with 1 ml. trypsin solution and 4 ml. buffer, each of 2 days' duration. Subsequent incubations with trypsin, followed by activated papain at pH 5 and pepsin at pH 1–2, removed no more than traces of N. Measurements of free α-amino-acid N in the autolysate and trypsin digests showed that about 90% of the total protein had been extracted as amino acid, while acid hydrolysis indicated that the residue after water and ethanol washings contained about 15% of the original protein content of the mycelium. 10–15% of the mycelium protein is therefore resistant to enzymic digestion. Acid hydrolysates (15 hr. in 6 N-HCl at 100°) of gelatin, stored mycelium, alkali-extracted protein, M_A (Table 1), and the protease-resistant residue, after removal of volatile acid by distillation *in vacuo* and neutralization, were tested in the Warburg apparatus with a preparation of D-amino-acid oxidase from sheep kidney, using the methods given by Bender & Krebs (1950). In each case (using about 5 mg. hydrolysed protein/sample) not more than 10 μl. O_2 was absorbed in 2 hr., whereas the addition of 1·0 mg. DL-alanine to a hydrolysate sample brought about an uptake of 60 μl. Protease resistance of mycelium preparations is thus not due to an accumulation of D-amino-acid residues.

Chitin. Like the carbohydrase activity, chitinase activity is probably diminished on air drying. The chitin of the mycelium is thus more resistant than the protein to autolysis. There is still, however, slight chitinase activity in the stored mycelium and 10% of the chitin N was found in solution as *N*-acetylglucosamine, where its presence was confirmed by paper chromatography.

The digestion of mycelial chitin was studied using the digests prepared for the breakdown of carbohydrate. Enzyme 19 AP, and preparations from *A. aureus* and *P. digitatum* had feeble chitinase activity compared with snail digestive juice. After five incubations with this last, from 80 to 90% of the chitin N was dispersed as *N*-acetylglucosamine. Subsequent incubations brought out no more, although accurate estimations are difficult when only small amounts are in solution, owing to the relatively high apparent *N*-acetylglucosamine content of the snail-juice blanks (about 10 μg. *N*-acetylglucosamine N/ml.). HCl hydrolysates were therefore prepared from the undigested residues and the deposit formed in the snail enzyme blank and the glucosamine hydrochloride content estimated. The amount of net residual chitin in the samples was from 3 to 8% of the original. Although these figures have no great accuracy, they confirm that only about 10%, or maybe less, of the chitin resists digestion.

DISCUSSION

The results obtained give some general idea of the breakdown of mycelium of *P. griseofulvum* by enzyme action. The lysing power of the enzyme systems of the mycelium is so considerable that a preliminary study of the effect of autolysis on the mycelial composition is required. About 55% of the fresh air-dried mycelium was dispersed in this way, including 84% of the protein, and it is very probable that, had not the carbohydrases and chitinases suffered a loss in activity through drying, there would have been a similar dispersion of carbohydrate and chitin. These components, although relatively more stable than protein to autolysis, are readily dispersed by snail digestive juice, which is known to contain a powerful cellulase and chitinase. After several incubations all but about 10% of the original chitin is dispersed and from 11 to 25% of the carbohydrate, the lower figure being obtained from data on the digestion of M_C, and the higher on the

apparently more resistant M_B (Tables 1 and 2). The reason for this discrepancy is not known. However, these figures show that about a tenth of the whole mycelium, including a part of each fraction analysed, is resistant to enzymic digestion or is not available to the enzyme. On the other hand, proteases, cellulases and chitinases are widespread in micro-organisms and invertebrates and their presence from these sources in the soil will in the absence of any stabilizing factors bring about the dispersion of the greater part of such fungal mycelium.

SUMMARY

1. Air-dried mycelium of *Penicillium griseofulvum* was subjected to autolysis. The suspended matter in the final product contained 16% of the original protein, 64% of the carbohydrate and 50% of the chitin. The solution contained dispersed protein and carbohydrate, but no α-amino acids or reducing sugar.

2. Air-dried mycelium after 2½–3 yr. storage was autolysed. The suspended material contained 39% of the original protein, 71% of the carbohydrate and 70% of the chitin. The solution contained amino acids, reducing sugar and *N*-acetylglucosamine.

3. The protease of the mycelium is stable to storage. Much polysaccharide-splitting and chitinase activity is lost on drying, but some of the residual activity remains after 3 yr. Such stored mycelium contains a very weak glucose oxidase, but no α-amino-acid oxidase, which was presumably very active in the fresh dry mycelium.

4. Little change takes place in the composition of the mycelium on storage except for a slight hydrolysis of protein.

5. 10–15% of the mycelium protein resists digestion with proteases; 10–25% of the carbohydrate and about 10% of the chitin resists digestion with juice from the alimentary tract of snails.

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REFERENCES

Bawden, F. C. & Pirie, N. W. (1946). *Brit. J. exp. Path.* **27**, 81.
Bender, A. E. & Krebs, H. A. (1950). *Biochem. J.* **46**, 210.
Benger, J., Johnson, M. J. & Petersón, W. H. (1937). *J. biol. Chem.* **117**, 429.
Bohonos, N., Woolley, D. W. & Peterson, W. H. (1942). *Arch. Biochem.* **1**, 319.
Dion, W. M. (1950). *Canad. J. Res.* C, **28**, 577.
Foster, J. W. (1949). *Chemical Activities of Fungi.* New York: Academic Press.
Holden, M. (1950). *Biochem. J.* **47**, 426.
Holden, M. & Tracey, M. V. (1950). *Biochem. J.* **47**, 421.
Michaelis, L. (1931). *Biochem. Z.* **234**, 139.
Morgan, W. T. J. & Elson, L. A. (1934). *Biochem. J.* **28**, 988.
Pirie, N. W. (1936). *Brit. J. exp. Path.* **17**, 269.
Smithies, W. R. (1952). *Biochem. J.* **51**, 259.
Smithies, W. R. (1953). *Biochem. J.* **53**, xxix.
Tracey, M. V. (1948). *Biochem. J.* **42**, 281.
Van Slyke, D. D., MacFadyen, D. A. & Hamilton, P. (1941). *J. biol. Chem.* **141**, 671.
Woolley, D. W. & Peterson, W. H. (1937). *J. biol. Chem.* **121**, 507.

The Oxidation of Phenylacetaldehyde by Plant Saps

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Kenten & Mann (1951) found that the total oxygen uptake obtained when pea-seedling extracts catalysed the oxidation of β-phenylethylamine was in excess of that required for the oxidation of the amine to phenylacetaldehyde. This suggested the presence of an aldehyde oxidase in the extracts. In accordance with this view it was found that large increases in the oxygen uptake were produced when phenylacetaldehyde was added to pea-seedling extracts. The oxidation products were not determined, although preliminary tests suggested that the oxidation was accompanied with hydrogen peroxide formation. By analogy with the known aldehyde oxidases phenylacetic acid was presumed to be the oxidation product. Since Skoog (1937) and Gordon & Sanchez-Nieva (1949) had obtained evidence that tryptamine could function as a precursor of 3-indolylacetic acid in plants, Kenten & Mann (1951) suggested that the successive action of amine oxidase and the aldehyde oxidase on tryptamine was a possible mechanism for the formation of 3-indolylacetic acid.

The present work was undertaken to investigate the system in pea-seedling extracts responsible for the oxidation of phenylacetaldehyde and to determine the oxidation products.

MATERIALS AND METHODS

Plant sap. Most of the plants examined were glasshouse-grown in a John Innes potting compost. The rest of the plants were from gardens in the neighbourhood of Rothamsted. The plant material was washed free from soil and then either ground in a mortar with sand or passed through a triple roller mill (Bawden & Pirie, 1944). The pulp was squeezed by hand through madapollam and centrifuged for 20–30 min. at 8000 rev./min. Pea-seedling sap was normally stored at 0° overnight and then centrifuged again. This treatment reduced the O_2 uptake of the sap without significant loss of activity towards the phenylacetaldehyde.

Peroxidase. Peroxidase preparations were made from horseradish by Mann's (1953) method. The purpurogallinzahl (P.Z.) (Willstätter & Stoll, 1918) of the preparations was estimated by Keilin & Mann's (1937) method. In the text the peroxidase activity of some preparations is given as purpurogallin (P.G.) units. A P.G. unit is the number of mg. of purpurogallin formed by 1 ml. of the preparation under Keilin & Mann's conditions.

Catalase. This was prepared from horse liver by a method based on that of Agner (1938) and the activity of the preparation was estimated as described previously (Kenten & Mann, 1952). The Katalasefähigkeit (Kat.f.) was 5000. Using the conversion factor of Chance & Herbert (1950) that Kat.f. $= 520\ k_1/M$, where M is the molecular weight of catalase (230000), $k_1 = 2{\cdot}2 \times 10^6\ \text{M}^{-1}\ \text{sec.}^{-1}$. For each experiment a sample of the preparation was suspended in 0·2 M-orthophosphate at pH 7 (10 mg./ml.) and the insoluble material removed by centrifuging.

Substrates. Samples of phenylacetaldehyde from L. Light and Co. Ltd. and A. Boake Roberts and Co. Ltd. were redistilled twice *in vacuo*, the head and tail fractions being discarded. The middle fraction was stored at 0° to reduce the rate of polymerization; even at this temperature slow polymerization occurred and distillation *in vacuo* at about fortnightly intervals was necessary to keep the content of polymer low. 1-Naphthylacetaldehyde was prepared from 2-(1-naphthyl)-1:1-di(ethoxycarbonylamino)ethane by the method of Jensen & Christensen (1950).

Methods. Carbonyl compounds were estimated by the bisulphite method of Clift & Cook (1932) and formic acid was estimated by Pirie's (1946) method. Absorption spectra were determined using a Unicam Model S.P. 500 quartz spectrophotometer.

Manometric measurements. These were carried out in the Warburg apparatus at 25°. The volume of the reaction mixture was 3 ml. KOH was omitted from the centre cup unless otherwise stated. CO_2 formation was measured by Warburg's direct method (Dixon, 1943).

EXPERIMENTAL AND RESULTS

Manometric studies of the oxidation of phenylacetaldehyde by plant saps

The addition of phenylacetaldehyde to most of the plant saps used caused an increase in oxygen uptake (Table 1). The rate of oxygen uptake was considerably reduced by previous heating of the pea-seedling sap at 100° (Fig. 1). The effect of heat was somewhat variable, but in general, while 5 min. at 100° reduced the activity to about 20% of the original activity, 20 min. at 100° did not completely inactivate the extract.

Specificity. Formaldehyde, acetaldehyde, glyoxal, *n*-butyraldehyde, *iso*valeraldehyde, cinnamaldehyde and phenylpropionaldehyde were tested with pea-seedling sap at a concentration of 10 mg./ml. under conditions similar to those used for Table 1. 1-Naphthylacetaldehyde was tested at a lower concentration (3·4 mg./ml.). In a period of 2 hr. only 1-naphthylacetaldehyde and *iso*butyraldehyde significantly increased the oxygen uptake, the effect being large with the former and small with the latter substrate.

Effect of dialysis and protein precipitants. Precipitates of pea-seedling sap, obtained by addition of ammonium sulphate to saturation, and dialysed sap (48 hr. at 0° against 200 vol. of water) catalysed the oxidation of phenylacetaldehyde by oxygen though at a much slower rate than an equivalent amount of untreated sap. Precipitates obtained by addition of

Table 1. *The oxidation of phenylacetaldehyde by plant saps*

(Reaction mixtures consisted of phenylacetaldehyde (10 mg. in 0·1 ml. ethanol) in 0·067 M-orthophosphate at pH 7. Plant sap (0·2–0·5 ml.) was added from the side arm. Blank O_2 uptakes were obtained using mixtures of ethanol (0·1 ml.), orthophosphate and plant sap.)

Plant	Part	Increased O_2 uptake (μl./ml. sap in 1 hr.)	Blank O_2 uptake (μl./ml. in 1 hr.)
Dandelion (*Taraxacum officinale* Weber)	Root	40	35
Lupin (*Lupinus*, garden hybrids)	Root	70	15
Potato (*Solanum tuberosum* L.)	Tuber	170	33
Pea seedling (*Pisum sativum* L.)	Root	2100	22
	Stem	1300	16
	Leaf	670	20
Tobacco (*Nicotiana tabacum* L.)	Leaf	700	16
Red clover (*Trifolium pratense* L.)	Leaf	260	18

2 vol. of acetone or ethanol to sap at 0° were more active than dialysed extract, but dialysis (48 hr. at 0° against 200 vol. of water) of water suspensions of these precipitates caused a considerable loss of activity (Table 2). These results suggested that the system responsible for the oxidation consisted of a non-dialysable factor which was precipitated by the precipitants used, and a dialysable factor not precipitated by ammonium sulphate, but at least in part by acetone and ethanol. In agreement with this it was found that the addition of protein-free filtrates of pea-seedling sap to dialysed sap, dialysed ethanol or acetone precipitates, or ammonium sulphate precipitates caused a considerable increase in activity (Table 2).

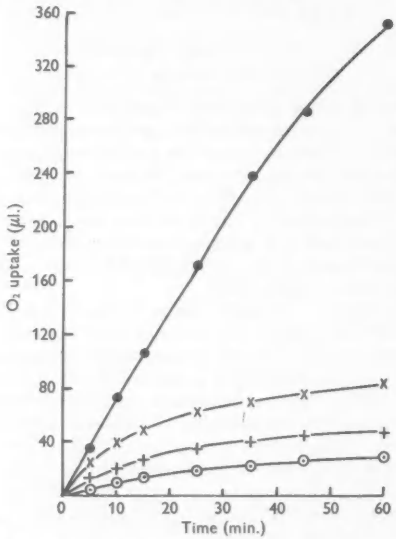

Fig. 1. The effect of previous heating of pea-seedling sap on the rate of oxidation of phenylacetaldehyde. Reaction mixtures as in Table 1; sap (0·2 ml.) previously heated at 100° for: ●—●, 0 min.; ×—×, 5 min.; +—+, 10 min.; ⊙—⊙, 20 min. The O_2 uptakes are corrected for the uptake in absence of phenylacetaldehyde.

Effect of ultrafiltration. Pea-seedling sap which had been kept at 0° overnight was centrifuged at 8000 rev./min. for 30 min. to remove chloroplast material and 30 ml. of the supernatant were filtered overnight at 0° in an ultrafiltration apparatus of the type described by Paterson, Pirie & Stableforth (1947). In this time nearly all the liquid passed through leaving a pasty residue inside the cellophan sac which was suspended in water (30 ml.). The oxygen uptake brought about by the addition of these fractions to phenylacetaldehyde is shown in Fig. 2. While the ultrafiltrate alone showed little

Table 2. *Effect of addition of ultrafiltrate on the oxidation of phenylacetaldehyde by fractions obtained from pea sap*

(Reaction mixtures as in Table 1; the various fractions (0·2 ml.) were added from the side arm alone or together with 0·2 ml. of ultrafiltrate. The O_2 uptake in the first hour was measured. Correction has been made for blank O_2 uptakes in mixtures from which phenylacetaldehyde, but not ethanol, was omitted. These were less than 10 μl. O_2 in 1 hr.)

Fraction	Uptake of O_2 (μl. in 1 hr.) Alone	Ultrafiltrate added
Pea sap	408	487
Dialysed pea sap	103	400
Ethanol precipitate	249	385
Dialysed ethanol precipitate	64	316
Acetone precipitate	327	418
Dialysed acetone precipitate	74	349
Saturated ammonium sulphate precipitate	134	359
Ultrafiltrate	12	—

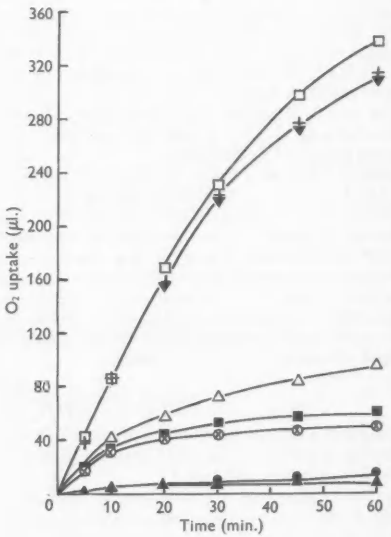

Fig. 2. The effect of ultrafiltration on the rate of oxidation of phenylacetaldehyde by pea-seedling sap. Reaction mixtures as in Table 1; after equilibration 0·2 ml. each of the following added from side arm: □—□, sap; ■—■, sap, heated; △—△, residue; ▲—▲, residue, heated; ●—●, ultrafiltrate; +—+, residue + ultrafiltrate; ⊗—⊗, residue, heated + ultrafiltrate; ▼—▼, residue + heated ultrafiltrate. Heat treatment was 15 min. at 100°.

activity and the residue alone only about one-third of the activity of the sap, together they gave an activity of the same order as sap. The effect of previous heating of the ultrafiltrate and the residue at 100° for 15 min. is also shown in Fig. 2. Whereas only slight activity remained in the residue after heating, the activating effect of the ultrafiltrate was not affected by heat treatment. Some ultrafiltrates had an activity of 30–60 μl. oxygen/0·2 ml./hr. with phenylacetaldehyde; this was presumably due to slight contamination by leakage during filtration since the activity was considerably reduced by heating for 15 min. at 100°.

The results suggest that the oxidation of phenylacetaldehyde by the pea-seedling sap is brought about by a thermolabile factor which requires the addition of a thermostable factor for maximal activity.

The nature of the thermostable factor. The activating effect of the ultrafiltrate was largely destroyed by ashing. However, acid extracts of the ash, after neutralization, had a slight activating effect when added to the residue in small amounts, but inhibited in larger amounts (Table 3). The effect of adding Co^{2+}, Ni^{2+}, Mn^{2+}, Hg^{2+}, Zn^{2+} and Cu^{2+} on the oxidation of phenylacetaldehyde by the residue was tested. At the concentrations used (0·001 M) only Mn^{2+} produced an increase in the rate of oxidation, while Cu^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+} reduced the rate, the effect being most marked with Cu^{2+} which caused a 50 % decrease. The activating effect of Mn^{2+} on the residue is also shown in Table 3. The rate of oxygen uptake increased with increasing amounts of Mn^{2+}, but the addition of 22 μg. Mn^{2+} did not increase the rate as much as the addition of ultrafiltrate containing 0·47 μg. Mn^{2+}. The fact that the addition of ultrafiltrate ash containing 1–2 μg. Mn^{2+} had little effect on the activity of the residue suggested that the ash contained an inhibiting factor. When ultrafiltrate was taken to dryness at 100° and then heated for 24–48 hr. at this temperature, 20–30 % of the activating effect was lost.

Table 3. *Effect of ultrafiltrate ash on the rate of oxidation of phenylacetaldehyde by pea-sap residue*

(Ultrafiltrate (15 ml.) was ashed and the ash extracted with N-HCl. The extract was neutralized and diluted to 15 ml. Mn content, determined by periodate oxidation, was 2·3 μg./ml. Reaction mixtures as in Table 1 with varying additions of ash solution or $MnSO_4$ (0·0002 M); pea-sap residue (0·2 ml.) was added from the side arm. The O_2 uptake in the first hr. was measured. Correction has been made for O_2 uptakes of blanks which were less than 10 μl. in 1 hr.)

Addition	Mn content (μg.)	Uptake of O_2 (μl. O_2 in 1 hr.)
None	—	118
Ash solution	0·23	144
	0·46	149
	0·92	125·5
	1·84	109·5
$MnSO_4$	0·55	167
	2·75	217
	22·0	291
Ultrafiltrate	0·47	443

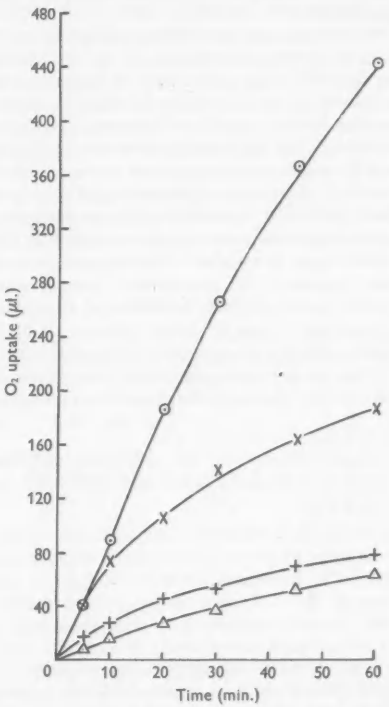

Fig. 3. The effect of catalase on the rate of oxidation of phenylacetaldehyde by pea-seedling sap. Reaction mixtures as in Table 1 with addition of catalase; pea-seedling sap (0·2 ml.) added after equilibration. Catalase added; ⊙—⊙, none; ×—×, 0·2 mg.; +—+, 1 mg.; △—△, 5 mg.

The effluent obtained when ultrafiltrate was passed three or four times through a column of the acid form of Zeo-Karb 215 resin had little effect on the activity of pea-sap residue. Over 90 % of the manganese contained in the ultrafiltrate was removed by the resin, but addition of Mn^{2+} to the effluent failed to restore its activity. If the resin used to deionize the ultrafiltrate was washed with N-hydrochloric acid, an eluate containing most of the manganese and 50–60 % of the original activity was obtained. Addition of effluent or Mn^{2+} to this eluate failed to increase its activity if allowance was made for the effect of Mn^{2+} alone.

The results suggest that at least part of the activating effect of ultrafiltrate is due to a factor(s) which is positively charged in weakly acid solution. If manganese is involved, it seems likely that either a cationic complex of manganese, or other inorganic or organic cations in addition to manganese, are responsible for the activating effect of ultrafiltrate on the residue.

Effect of catalase. The effect of catalase on the oxidation of phenylacetaldehyde by pea-seedling sap is shown in Fig. 3. The rate of oxygen uptake decreases with the increasing catalase addition, a concentration of 5 mg./3 ml. reducing the rate by about 85 %. The inhibiting effect of catalase suggested that the reaction studied depended on the production of hydrogen peroxide and was a peroxidatic oxidation, since with a simple flavoprotein enzyme system addition of catalase would, at most, halve the rate of uptake. It is known that manganese together with peroxidase forms a system capable of catalysing the oxidation of a number of substrates by oxygen (Kenten & Mann, 1953). The activating effect of manganese on the residue and the inhibition by catalase suggested that peroxidase might be the thermolabile factor in pea-seedling sap.

Manometric studies of the oxidation of phenylacetaldehyde by horseradish peroxidase and manganous ions

Horseradish-peroxidase preparations catalyse the oxidation of phenylacetaldehyde and the rate of oxidation is increased by the addition of ultrafiltrate or Mn^{2+}. This is shown by the results of Fig. 4. Control experiments with reaction mixtures from which phenylacetaldehyde but not ethanol was omitted gave negligible oxygen uptakes. The oxygen uptake with peroxidase alone was small and was only 30–35 % of that given by pea-sap residue of approximately the same peroxidase content. The greater activity of the residue is most likely due to the presence of small amounts of the natural activating factor. The rate of oxygen uptake increased markedly on addition of Mn^{2+} or ultrafiltrate. The increased uptake on addition of ultrafiltrate containing 0·27 μg. Mn^{2+} was considerably greater than that obtained by the addition of 2·75 μg. Mn^{2+}, showing that, as found with pea-sap residue, Mn^{2+} alone cannot be the natural activating factor in ultrafiltrate.

Effect of catalase. This was tested in reaction mixtures with varying additions of catalase. The resulting oxygen uptakes are shown in Fig. 5. The inhibiting effect of catalase is similar to that obtained when pea-seedling sap is used to bring about the oxidation (Fig. 3).

Comparison of pea-sap residue and horseradish peroxidase. Pea-sap residue has high peroxidase activity; under the standard conditions (Keilin & Mann, 1937) with pyrogallol as substrate the P.Z. of different preparations varied from 5 to 30.

The activating effect of Mn^{2+} on a pea-sap residue of P.Z. 25 and a horseradish peroxidase of P.Z. 485 was compared in the following way. After equilibration, samples of residue and horseradish peroxidase equivalent to 7 P.G. units were added from the side arm to a mixture containing phenylacetaldehyde and varying amounts of Mn^{2+} in orthophosphate at pH 7. The oxygen uptakes in the first hour are recorded in Table 4. While both the horseradish peroxidase and the residue gave increased uptakes in the presence of Mn^{2+}, the horseradish peroxidase gave larger increases for a given Mn^{2+} addition than did the pea residue.

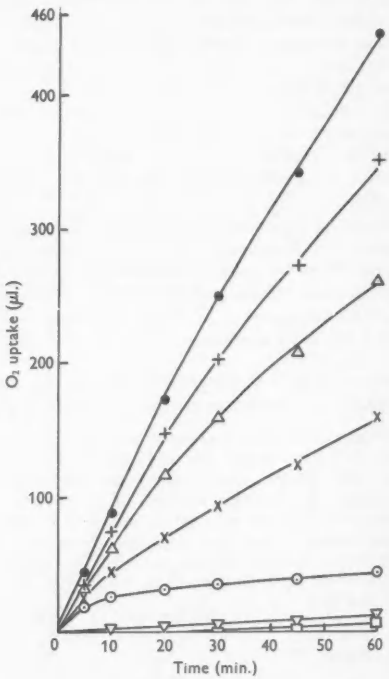

Fig. 4. The effect of Mn^{2+} and ultrafiltrate on the rate of oxidation of phenylacetaldehyde by horseradish peroxidase. Reaction mixtures as in Table 1 with varying additions of $MnSO_4$ or 0·2 ml. of ultrafiltrate; 0·2 ml. horseradish peroxidase of P.Z. 485 (65 μg./ml.) added from side arm. ⊙—⊙, ×—×, △—△, +—+, peroxidase with 0, 0·22, 0·55 and 2·75 μg. Mn^{2+}, respectively; ●—●, peroxidase + ultrafiltrate; ▽—▽, ultrafiltrate; □—□, 2·75 μg. Mn^{2+}.

Effect of pH. When horseradish peroxidase plus Mn^{2+} catalysed the oxidation of phenylacetaldehyde in phosphate-borate buffers the rate of oxygen uptake increased with increase in pH value from 6 to 9. At pH values above 9 freshly made solutions of phenylacetaldehyde went rapidly turbid, the change being more rapid the higher the pH. Aldehyde estimations showed that this change was accompanied by a decrease in the total aldehyde content of the solutions. This change (which was presumably due to polymerization of the aldehyde, possibly by an aldol condensation) was small at pH 7·5 where the total aldehyde content of 0·01 M-phenylacetaldehyde decreased less than 5% in 8 hr. Accordingly, although the activity of the system was less at pH 7–7·5 than at more alkaline pH, the experiments of the present work have been carried out at pH 7–7·5 to avoid the loss of substrate which takes place at higher pH values.

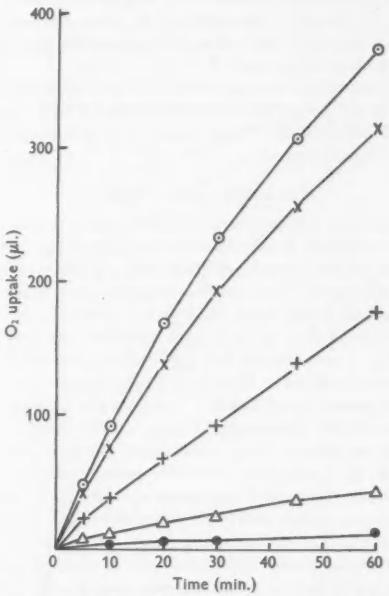

Fig. 5. The effect of catalase on the rate of oxidation of phenylacetaldehyde by horseradish peroxidase plus Mn^{2+}. Reaction mixtures as in Table 1 with addition of 2·75 μg. Mn^{2+} and varying amounts of catalase; 0·2 ml. horseradish peroxidase of P.Z. 485 (70 μg./ml.) added from side arm. Catalase added: ⊙—⊙, none; ×—×, 0·2 mg.; +—+, 1 mg.; △—△, 5 mg.; ●—●, 5 mg. catalase, peroxidase omitted.

Table 4. *Comparison of pea-sap residue and horseradish peroxidase*

(Reaction mixtures as in Table 1 with varying amounts of $MnSO_4$. After equilibration 0·2 ml. of pea-sap residue of P.Z. 25 (34·5 P.G. units/ml.), or 14·2 μg. horseradish peroxidase of P.Z. 485 in 0·2 ml. of water, was added from the side arm. Correction has been made for O_2 uptakes of blanks which were less than 10 μl. in 1 hr.)

Mn^{2+} added (μg.)	Uptake of O_2 (μl. in 1 hr.) Residue	Horseradish peroxidase
0	127	32
0·55	191	236
2·75	235	311
22·0	232	384

Effect of substrate concentrations. The effect of substrate concentration on the reaction velocity was tested with pea-seedling sap and horseradish peroxidase plus Mn^{2+} in 0·067 M orthophosphate at pH 7. The initial velocity increased markedly with increase in substrate concentration (Table 5).

Table 5. *The effect of substrate concentration*

(Reaction mixtures as in Table 1, with 0·5 ml. of 0·0001 M-$MnSO_4$ (2·75 μg. Mn^{2+}) where peroxidase was used. After equilibration 0·2 ml. of pea-seedling sap, or 20 μg. of horseradish peroxidase of P.Z. 485 in 0·2 ml. of water, was added from the side arm.)

Phenylacetaldehyde concentration (mg./3 ml.)	Increased O_2 uptake (μl. in 10 min.) Sap	Peroxidase and Mn^{2+}
10	75·5	72
5	67	64
1	15	20·5
0·5	5·5	11
0·1	1	4·5
0·05	0·5	2·5

Maximum velocity was not reached at a concentration of 5 mg./3 ml. where not all the phenylacetaldehyde was in solution. This suggested that the rate of oxidation was limited by the solubility of the phenylacetaldehyde; increase in the amount of phenylacetaldehyde by increasing the rate of solution would help to maintain saturation and therefore increase the velocity of the reaction.

Oxidation of Mn^{2+} *during the oxidation of phenylacetaldehyde by peroxidase.* Kenten & Mann (1949, 1950) have shown that the peroxidase-catalysed oxidation of Mn^{2+} by hydrogen peroxide depends on the presence of a phenolic substrate, such as *p*-cresol. In pyrophosphate at pH 7 it was shown that a pink manganipyrophosphate complex was formed. The manganipyrophosphate was estimated manometrically with hydrazine by measuring the evolution of nitrogen.

If the oxidation of phenylacetaldehyde by peroxidase plus Mn^{2+} is carried out in pyrophosphate at pH 7 the reaction mixture develops a pink colour suggesting the formation of manganipyrophosphate.

The composition of the reaction mixtures and results are given in Table 6. On adding the peroxidase from the side arm there was a short lag of about 2–3 min. before the oxygen uptake commenced both with the complete reaction mixture and where *p*-cresol was omitted. Whereas the presence of *p*-cresol with peroxidase in the reaction mixture from which Mn^{2+} had been omitted considerably reduced the rate of oxygen uptake, in the presence of Mn^{2+} the inhibiting effect of *p*-cresol was negligible.

Table 6. *The oxidation of manganese during the oxidation of phenylacetaldehyde by peroxidase*

(Reaction mixtures consisted of 0·1 ml. 0·2 M-phenylacetaldehyde, 0·2 ml. 0·1 M-$MnSO_4$ and 0·3 ml. 0·001 M-*p*-cresol in 0·33 M-pyrophosphate at pH 7. After equilibration 50 μg. horseradish peroxidase of P.Z. 485 in 0·2 ml. of water were added from the side arm. Two hr. after the peroxidase addition the manometers were gassed with N_2 and 0·2 ml. saturated hydrazine sulphate was added from the second side arm.)

Substance omitted from reaction mixture	Uptake of O_2 (μl.)	Output of N_2 with hydrazine (μl.)
None	346	28·5
p-Cresol	349	24
$MnSO_4$	75	6
Peroxidase	38	4
$MnSO_4$ and *p*-cresol	127·5	4
p-Cresol and peroxidase	49·5	6·5

Where Mn^{2+} was present in the absence of peroxidase a considerable oxygen uptake was obtained but no pink coloration was observed in the reaction mixtures. The Mn^{2+}, at the high concentration used, catalyses the autoxidation of the aldehyde but does not lead to the accumulation of manganipyrophosphate. With both the complete reaction mixture and that from which *p*-cresol was omitted a pink colour developed and appeared to increase in intensity as the reaction proceeded. After gassing with nitrogen the addition of hydrazine discharged this pink colour and the largest gas output (presumably nitrogen, Kenten & Mann, 1949) was obtained from the complete reaction mixture and the mixture from which *p*-cresol was omitted. These results suggest that oxidation of Mn^{2+} can take place during the reaction and leads to the accumulation of manganipyrophosphate. The accumulation of oxidized manganese was, however, small being equivalent to 0·28 and 0·24 mg. Mn^{3+} in the complete reaction mixture and in the mixture from which only *p*-cresol was omitted, respectively. Since phenylacetaldehyde slowly reduces manganipyrophosphate at pH 7 the amounts of manganipyrophosphate formed are probably somewhat greater than those found experimentally.

Specificity. The aldehydes previously tested with pea-seedling sap were tested at a concentration of 3 mg./ml. with horseradish peroxidase of P.Z. 485 and 2·75 μg. Mn^{2+} in orthophosphate at pH 7. In 2 hr. a small oxygen uptake was obtained with *iso*-butyraldehyde. Under the conditions used for Fig. 6, 1-naphthylacetaldehyde was oxidized by peroxidase and Mn^{2+}, but with this substrate there was a short lag period of about 5–10 min. before the oxygen uptake started and the rate of oxidation was less than with phenylacetaldehyde. Peroxidase in the absence of Mn^{2+} catalysed the oxidation though at a slower rate.

The course of the oxidation

A study has been made of the oxygen uptake and the oxidation products when pea-seedling sap and horseradish peroxidase plus Mn^{2+} oxidize phenylacetaldehyde. The results suggest that benzaldehyde and formic acid are two of the products while the formation of hydrogen peroxide as an obligatory intermediate is suggested by the inhibiting effect of catalase. Most of the results have been got using peroxidase plus Mn^{2+} as catalyst of the oxidation. With pea-seedling sap, particularly when large amounts of sap were used, aldehyde estimations by bisulphite titration were unsatisfactory owing to the large blank and indefinite end point.

Oxygen uptake and carbon dioxide output. With the amounts of pea-seedling sap and horseradish peroxidase used, the initial rate of oxygen uptake was rapid until a value approaching 1 mol. O_2/mol. phenylacetaldehyde was reached, when the rate of uptake fell off rapidly. Generally, 4–6 hr. was required for the total uptake to reach 0·9–1 mol. oxygen/mol. aldehyde. The oxygen uptake then continued at a slow rate varying between 4 and 10 μl. oxygen/hr. in different experiments. Increasing the amount of pea sap or peroxidase and Mn^{2+} present initially or adding second amounts from the side arm after the initial rapid uptake was complete gave increases of only 5–10 μl. oxygen in 6 hr. Typical curves showing the rate of oxygen uptake are shown in Fig. 6.

It would appear that the first stage of the reaction is enzyme catalysed and leads to the consumption of 1 mol. oxygen/mol. phenylacetaldehyde. The secondary slow oxygen uptake is presumably due to further oxidation of the primary reaction products and in view of the rate may not be enzyme catalysed.

Small amounts of carbon dioxide were apparently produced. Using Warburg's direct method the final respiratory quotients determined at oxygen uptakes between 0·9 and 1 mol. oxygen/mol. phenylacetaldehyde were 0·05–0·1 with pea-seedling sap and 0·02–0·04 with horseradish peroxidase and Mn^{2+}. Where potassium hydroxide was present in the inner cup slight yellowing of the filter-paper wick showed that some aldehyde had been

lost from the solution. Control experiments with phenylacetaldehyde in orthophosphate at pH 7·5 in the main vessel showed that this loss could amount to 5–10 % in 6 hr. Since the oxidation proceeds rapidly in the initial stages the actual loss during the enzyme reaction was probably less than this.

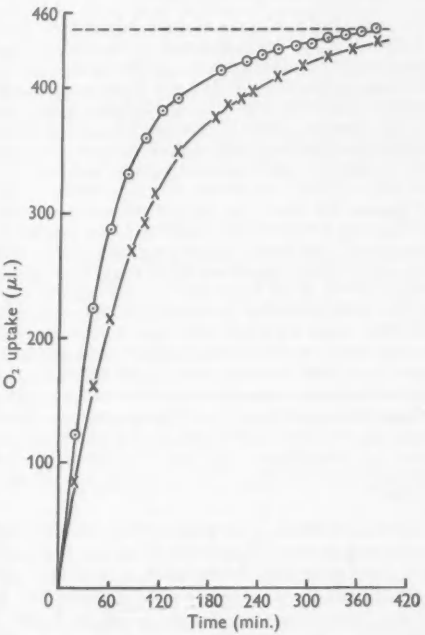

Fig. 6. The oxidation of phenylacetaldehyde by pea-seedling root sap and horseradish peroxidase plus Mn^{2+}. Reaction mixtures consisted of 0·1 ml. of 0·2 M-phenylacetaldehyde in ethanol in 0·067 M orthophosphate at pH 7·5 and 0·5 ml. of 0·0001 M-$MnSO_4$ where peroxidase was used. After equilibration 0·5 ml. of pea-seedling root sap or 100 µg. of horseradish peroxidase of P.Z. 485 in 0·1 ml. of water were added from the side arm. KOH was present in the inner cup. ⊙—⊙, Sap; ×—×, peroxidase plus Mn^{2+}. The dotted line shows the uptake for 1 mol. O_2/mol. aldehyde.

The formation of benzaldehyde. The oxidation of phenylacetaldehyde by pea-seedling sap and horseradish peroxidase plus Mn^{2+} is accompanied by the formation of a product giving an absorption maximum at 250 mµ. A typical set of curves obtained, using horseradish peroxidase plus Mn^{2+}, are shown in Fig. 7. In this experiment a reaction mixture identical to that used for Fig. 6 was shaken in air at 25°. Samples (0·4 ml.) were pipetted at intervals into 24·6 ml. of water at 0° and the ultraviolet-absorption curve of this solution was determined, using in the blank cell a suitably diluted mixture from which phenylacetaldehyde had been omitted.

In other experiments with a similar reaction mixture on a larger scale, the reaction was stopped when the O_2 uptake had reached a value of about 1 mol. O_2/mol. aldehyde, by cooling the mixture to 0°. Steam distillation of 0·5 ml. portions in the Markham (1942) still was carried out collecting exactly 50 ml. distillate. The ultraviolet-absorption curve was determined using a distillate from a suitable enzyme-phosphate mixture in the blank cell. The absorption curve of these distillates was very similar to that given by benzaldehyde in water, Fig. 8. That benzaldehyde is a product of the oxidation was confirmed by the isolation of benzaldehyde 2:4-dinitrophenylhydrazone from large-scale reaction mixtures (p. 358).

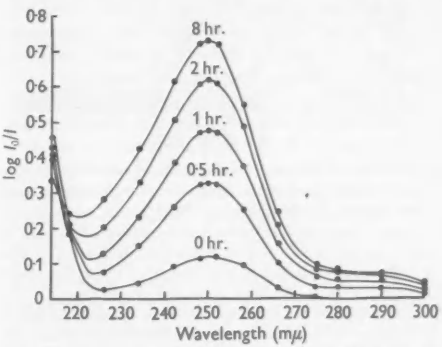

Fig. 7. The change in the ultraviolet-absorption spectrum when phenylacetaldehyde is oxidized by horseradish peroxidase plus Mn^{2+}. Reaction mixture as in Fig. 6.

Further proof that benzaldehyde was the product causing most of the absorption at 250 mµ. was shown by the change in the absorption curve to one closely resembling benzoic acid when the final reaction mixture was treated with Ag_2O. Many aldehydes are quantitatively oxidized to the corresponding acid by this reagent (Bailey & Knox, 1951; Smith & Mitchell, 1950).

One ml. of final reaction mixture and 1 ml. of a suspension of freshly precipitated Ag_2O (50 mg./ml.) were incubated at 37° overnight. After centrifuging, 1 ml. of the supernatant, together with 3 ml. 4 N-H_2SO_4, was steam distilled and 50 ml. distillate collected. The absorption curve determined against a suitable blank distillate was practically identical with that of a solution of benzoic acid in water (Fig. 8). It was found by measuring the absorption at 250 mµ. that benzaldehyde solutions in water obeyed Beer's Law over the range 1·5–6 µg./ml. and log ϵ was 4·10. Furthermore, at 226 mµ. log ϵ of benzoic acid in water was found to be 3·97 at a concentration of 5·6 µg./ml. These results permit the following calculations. Assuming the absorption at 250 mµ. is entirely due to benzaldehyde, then the concentration of benzaldehyde would be 0·37 mg./ml. in the final reaction mixture and 0·35 mg./ml. in the steam distillate. From the absorption at 226 mµ. after Ag_2O treatment and steam distillation, 0·46 mg. benzoic acid/ml. is present suggesting the presence of 0·40 mg. benzaldehyde/ml. in the final reaction mixture. If the contents of the still remaining after

the initial steam distillation of the neutral reaction mixtures are acidified with 3 ml. 4N-H_2SO_4 and distillation is continued until a further 50 ml. distillate is obtained the absorption curve of this solution (Fig. 8) suggests that 0·11 mg./ml. of benzoic acid may be present in the final reaction mixture. The value obtained above for benzoic acid formed by the Ag_2O treatment is, therefore, too high. The corrected value would be 0·35 mg. benzoic acid/ml., which is equivalent to 0·30 mg. benzaldehyde/ml.

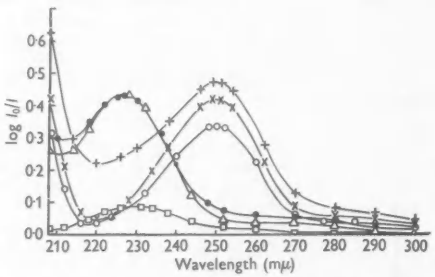

Fig. 8. Ultraviolet-absorption spectra of reaction mixture of horseradish peroxidase, Mn^{2+} and phenylacetaldehyde after steam distillation and treatment with Ag_2O. +—+, Reaction mixture; ×—×, steam distillate of reaction mixture; ○—○, benzaldehyde 70 μg./25 ml.; ●—●, steam distillate of acidified Ag_2O-treated reaction mixture; △—△, benzoic acid 140 μg./25 ml.; □—□, steam distillate of acidified reaction mixture obtained subsequent to a preliminary distillation at neutral pH. Only a few of the experimental points are plotted for the sake of clarity.

The agreement between these values suggests that measurement of the absorption of reaction mixtures at 250 mμ. can be used to estimate (probably to ± 20 %) the amount of benzaldehyde present. The values given for the rate of benzaldehyde formation in Table 7 have been calculated using the absorption at 250 mμ. and are corrected for the absorption of the phenylacetaldehyde present, assuming that solutions of phenylacetaldehyde obey Beer's Law and that a molecule of phenylacetaldehyde is lost per molecule of benzaldehyde formed.

The formation of formic acid. The oxidation of phenylacetaldehyde to benzaldehyde requires the formation of an equivalent amount of a compound containing one carbon atom. The evidence suggests that this compound is formic acid.

The results for formic acid and steam-volatile acid formation given in Table 7 were obtained in the following way. A reaction mixture of the same composition as that used for Fig. 6 was shaken in air at 25°, and 10 ml. samples were pipetted at intervals into 5 ml. of 4N-H_2SO_4 to stop the reaction. Portions (7 ml.) of these acidified samples were distilled in the Markham (1942) still collecting two successive 50 ml. of distillate. The distillates were titrated to pH 7·5 with 0·01N-$Ba(OH)_2$ to measure the total steam-volatile acid present. The first 50 ml. of distillate, which contained 80–85 % of the total acid, was concentrated and used for the estimation of formic acid. By distilling control mixtures of formic acid, orthophosphate and H_2SO_4 it was found that an average of 85 % of the formic acid was recovered in 50 ml. distillate when distillation was carried out from a volume of 7 ml. The values for formic acid have accordingly been corrected on this basis. The total steam-volatile acid finally present was some 20–30 % greater than the amount of formic acid present, suggesting that small amounts of other acids, possibly phenylacetic or benzoic acid, are formed during the reaction. Distillation of control mixtures of benzoic or phenylacetic acid with orthophosphate and H_2SO_4 gave recoveries 95 and 62 % respectively in 100 ml. of distillate.

Loss of aldehyde. The total amount of carbonyl compounds present in reaction mixtures containing phenylacetaldehyde, horseradish peroxidase and Mn^{2+} decreased as the oxygen uptake proceeded. In general, under the conditions given in Table 7, 25–35 % of the total carbonyl content was lost in 6–8 hr.

Isolation of benzaldehyde 2:4-dinitrophenylhydrazone. A reaction mixture containing 220 mg. phenylacetaldehyde, 4 ml. 0·001M-$MnSO_4$, 5 mg. horseradish peroxidase of P.Z. 485 and 100 ml. 0·2M-orthophosphate at pH 7·5, in a total volume of 300 ml. was shaken in air at room temperature for 8 hr. The mixture was left at 0° overnight and then filtered. Samples were removed at intervals and the benzaldehyde

Table 7. *Oxygen uptake and the formation of benzaldehyde, formic acid and steam-volatile acid during the oxidation of phenylacetaldehyde by peroxidase plus* Mn^{2+}

(The O_2 uptake was followed manometrically in reaction mixtures of 3 ml. consisting of 0·1 ml. 0·2M phenylacetaldehyde in ethanol, 0·5 ml. 0·0001M-$MnSO_4$ and 100 μg. horseradish peroxidase of P.Z. 485 in 0·067M orthophosphate, pH 7·5. Reaction mixtures of the same composition but on a larger scale were shaken in air at 25° and samples withdrawn at intervals for the estimation of benzaldehyde, formic acid, steam-volatile acid and total carbonyl compounds.)

Time (hr.)	Uptake of O_2 (μmoles)	Benzaldehyde (μmoles)	Formic acid (μmoles)	Steam-volatile acid (μmoles)	Total carbonyl compounds (μmoles)
0	0	0	0·3	0·9	20·2
1	9·6	5·8	4·7	5·5	18·2
2	14·4	8·1	6·5	8·4	16·9
4	17·9	9·2	8·4	10·4	15·6
8	19·0	9·9	8·8	12·5	13·4

content estimated spectrophotometrically; such estimations showed a total of 67 mg. of benzaldehyde in the residual filtrate (263 ml.). To this filtrate 100 ml. of a saturated solution of 2:4-dinitrophenylhydrazone in 2N-HCl were added, and the mixture was allowed to stand for 15 min. The orange precipitate was centrifuged off, washed with water on the centrifuge, and then transferred with hot ethanol to a tared beaker. Removal of the ethanol and drying *in vacuo* gave 261 mg. of orange-brown product. This was suspended in boiling benzene and freed from an insoluble brown material by filtration. Concentration of the filtrate yielded 49·5 mg. of orange crystals, m.p. 229–230°. Mixed m.p. with an authentic sample of benzaldehyde 2:4-dinitrophenylhydrazone (m.p. 233–234°) was 231–232°. (All m.p.'s uncorr.) The yield based on benzaldehyde formed was 27%. A second crop of 22 mg. orange crystals, m.p. 203–208°, was obtained from the mother liquor.

DISCUSSION

The results of the present work show that many plant saps catalyse the oxidation of phenylacetaldehyde by oxygen. The system responsible for this oxidation consists of, in pea-seedling sap, a thermolabile and a thermostable factor. The evidence suggests that the thermolabile factor is a peroxidase. It can be replaced by horseradish-peroxidase preparations, and in agreement with the thermolabile properties of peroxidase reported by Gallagher (1924) and Herrlinger & Kiermeier (1944) prolonged heating of the natural system at 100° is necessary for inactivation. Furthermore, the inhibiting effect of catalase suggests that the reaction is peroxidatic. The thermostable factor can be partially replaced by Mn^{2+}, and using horseradish peroxidase plus Mn^{2+} it is possible to construct a system with similar properties to that in pea-seedling sap. The thermostable factor in pea sap, however, is not identical with Mn^{2+} since addition of this factor to peroxidase gives a system of much greater activity than addition of Mn^{2+} at the concentration contained in the thermostable factor. Although the thermostable factor in the natural system has not been identified, the experiments with ion-exchange resin suggest that the activity of this factor is partially dependent on a substance or substances which are positively charged in weakly acid solution, and it is possible that the activity is due to a cationic complex of manganese which is more effective than Mn^{2+}.

The mechanism of the reaction is obscure, but the formation of hydrogen peroxide as an obligatory intermediate is suggested by the inhibiting effect of catalase. Hydrogen peroxide accumulation during the reaction has not been demonstrated. Chance (1952) has suggested that a Mn^{2+}-activated peroxidase-peroxide complex is the catalyst for the oxidase reaction with dihydroxymaleic acid, peroxidase and Mn^{2+}, and it is possible that a similar complex catalyses the oxidation of phenylacetaldehyde. The results of Kenten & Mann (1953) suggested that the oxidation of certain dicarboxylic acids by peroxidase and Mn^{2+} was dependent on manganese oxidation. While manganese oxidation can be demonstrated when phenylacetaldehyde is oxidized by peroxidase in pyrophosphate at high Mn^{2+} concentrations, it may be that oxidation of manganese is a side reaction. Neither hydrated manganese dioxide nor manganipyrophosphate appear to oxidize phenylacetaldehyde rapidly at neutral pH and, if manganese oxidation plays a part in the reaction, the oxidized manganese must react with an intermediate product. In contrast to the peroxidase systems studied by Kenten & Mann (1950, 1952) manganese oxidation takes place in the absence of a phenolic compound such as *p*-cresol suggesting the formation either of a manganocomplex which is directly oxidized by peroxidase, or of phenolic compounds during the reaction. Manganese catalyses the autoxidation of phenylacetaldehyde though at a very slow rate unless present in high concentration. Such autoxidation if it leads to the production of hydrogen peroxide may accelerate the reaction.

The oxidation of phenylacetaldehyde by pea-seedling sap or horseradish peroxidase and Mn^{2+} proceeds with the formation of benzaldehyde and formic acid as two of the products. Under the experimental conditions used, the oxygen uptake is rapid until about 0·9 mol. oxygen/mol. aldehyde is consumed when the oxygen uptake falls to a low rate. When the oxygen uptake has reached a value of 0·9–1·0 mol. oxygen/mol. phenylacetaldehyde, approximately 0·5 mol. benzaldehyde has been formed per mol. phenylacetaldehyde initially present. Some phenylacetic acid may be formed since the values for steam-volatile acid are somewhat greater than can be accounted for as formic acid, but the amount is not sufficient to resolve the discrepancy of about 0·5 mol. between the phenylacetaldehyde initially present and the benzaldehyde formed. The loss of aldehyde which occurs as the reaction proceeds shows that the reaction does not consist only of the conversion of phenylacetaldehyde to benzaldehyde and formic acid.

Evidence has been obtained that 1-naphthylacetaldehyde is converted to 1-naphthylacetic acid by *Avena*-coleoptile juice (Larsen, 1951) and by *Artemisia*-seedling-root juice (Ashby, 1951). Larsen (1951) found 1 mol. acid was formed per 2 mol. aldehyde added and suggested that the conversion process might be a dismutation in which equivalent amounts of alcohol and acid were formed. Ashby (1951), however, found considerably less acid formation than 1 mol. per 2 mol. aldehyde and he concluded that the root juice was able to change 1-naphthylacetaldehyde in some manner other than by oxidation or a dismutation to 1-naphthylacetic

acid. The results of Ashby (1951) are in better agreement with the results of the present work, if it is assumed that the oxidation of phenylacetaldehyde and 1-naphthylacetaldehyde proceed in the same way. It has been shown in the present work that 1-naphthylacetaldehyde is oxidized by both pea sap and horseradish peroxidase plus Mn^{2+}. By analogy with phenylacetaldehyde it seems likely that 1-naphthylacetaldehyde and also 3-indolylacetaldehyde would be oxidized with the formation of 1-naphthaldehyde and 3-indolealdehyde, respectively.

There is indirect evidence that partial conversion of 3-indolylacetaldehyde into 3-indolylacetic acid takes place in certain intact plants and with certain plant extracts (Larsen, 1949; Gordon & Sanchez-Nieva, 1949; Ashby, 1951; Brown, Henbest & Jones, 1952). Since most plant extracts and crude plant-enzyme preparations contain peroxidase and Mn^{2+} the results of the present work suggest that *in vitro* studies of the metabolism of 1-naphthylacetaldehyde and 3-indolylacetaldehyde with such preparations may lead to the demonstration of partial conversion of these compounds to 1-naphthaldehyde and 3-indolealdehyde respectively.

It is not yet known how far the system responsible for the oxidation of phenylacetaldehyde is of significance *in vivo*. Phenylacetaldehyde is not a known plant constituent, but β-phenylethylamine is, and by the action of plant amine oxidase could form phenylacetaldehyde. The oxidation proceeds readily with both pea-seedling sap and at physiological concentrations of peroxidase and Mn^{2+} with relatively high substrate concentration, but only with difficulty at low substrate concentration. The reaction is of interest since, like the previously reported oxidations of certain dicarboxylic acids (Swedin & Theorell, 1940; Kenten & Mann, 1953), it shows that peroxidase may bring about oxidations independently of external sources of hydrogen peroxide. Peroxidase, together with the natural activating factor, may act as a direct oxidase system in the intact plant, and peroxidase action *in vivo* may, therefore, proceed in the absence of flavoprotein enzyme systems. As yet few compounds have been tested, and no well-known plant metabolite has been shown to be oxidized by peroxidase and Mn^{2+} under physiological conditions. However, preliminary results suggest that horseradish-peroxidase preparations alone will catalyse the oxidation of 3-indolylacetic acid by oxygen, while peroxidase plus Mn^{2+} is a more efficient catalyst of the reaction.

SUMMARY

1. Many plant saps oxidize phenylacetaldehyde. The system in pea-seedling sap consists of a thermolabile factor which appears to be a peroxidase and a thermostable factor which can be partially replaced by manganous ions. A system with similar properties to that in pea-seedling sap can be constructed using horseradish-peroxidase preparations and manganous ions.

2. The oxidation when catalysed by either pea-seedling sap or horseradish peroxidase and manganous ions proceeds with the formation of benzaldehyde and formic acid.

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REFERENCES

Agner, K. (1938). *Biochem. J.* **32**, 1702.
Ashby, W. C. (1951). *Bot. Gaz.* **112**, 237.
Bailey, H. C. & Knox, J. H. (1951). *J. chem. Soc.* p. 2741.
Bawden, F. C. & Pirie, N. W. (1944). *Brit. J. exp. Path.* **25**, 68.
Brown, J. B., Henbest, H. B. & Jones, E. R. H. (1952). *Nature, Lond.*, **169**, 335.
Chance, B. (1952). *J. biol. Chem.* **197**, 577.
Chance, B. & Herbert, D. (1950). *Biochem. J.* **46**, 402.
Clift, F. P. & Cook, R. P. (1932). *Biochem. J.* **26**, 1788.
Dixon, M. (1943). *Manometric Methods*, 2nd ed. p. 64. Cambridge University Press.
Gallagher, P. H. (1924). *Biochem. J.* **18**, 39.
Gordon, S. A. & Sanchez-Nieva, F. (1949). *Arch. Biochem.* **20**, 367.
Herrlinger, F. & Kiermeier, F. (1944). *Biochem. Z.* **317**, 1.
Jensen, K. A. & Christensen, S. A. K. (1950). *Acta chem. scand.* **4**, 703.
Keilin, D. & Mann, T. (1937). *Proc. Roy. Soc.* B. **122**, 119.
Kenten, R. H. & Mann, P. J. G. (1949). *Biochem. J.* **45**, 255.
Kenten, R. H. & Mann, P. J. G. (1950). *Biochem. J.* **46**, 67.
Kenten, R. H. & Mann, P. J. G. (1951). *Biochem. J.* **50**, 360.
Kenten, R. H. & Mann, P. J. G. (1952). *Biochem. J.* **52**, 125.
Kenten, R. H. & Mann, P. J. G. (1953). *Biochem. J.* **53**, 498.
Larsen, P. (1949). *Amer. J. Bot.* **36**, 32.
Larsen, P. (1951). *Plant Physiol.* **26**, 697.
Mann, P. J. G. (1953). Private communication.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Paterson, J. S., Pirie, N. W. & Stableforth, A. W. (1947). *Brit. J. exp. Path.* **28**, 223.
Pirie, N. W. (1946). *Biochem. J.* **40**, 100.
Skoog, F. (1937). *J. gen. Physiol.* **20**, 311.
Smith, J. & Mitchell, D. M. (1950). *Analyt. Chem.* **22**, 746.
Swedin, B. & Theorell, H. (1940). *Nature, Lond.*, **169**, 335.
Willstätter, R. & Stoll, A. (1918). *Liebigs Ann.* **416**, 21.

Chemical Synthesis of Hexose Polymers

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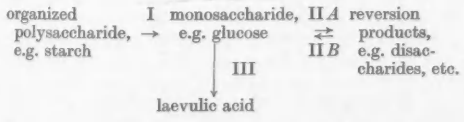
It has long been recognized that, under suitable conditions, disaccharides may be formed chemically from monosaccharides. Fischer (1890, 1898) reported that heating of glucose with concentrated hydrochloric acid resulted in the formation of a mixture of substances including disaccharides. This process, termed 'reversion', appeared to proceed under conditions favouring the reversal of the acid hydrolysis of polysaccharides. Drew & Haworth (1927) obtained a polymer of ten units by dehydration of 2:3:4-trimethyl arabonolactone, whilst more recently Deriaz (1949) and Overend, Shafizadeh & Stacey (1951) found that distillation of methyl furanoside of 2-deoxyribose and 2-deoxygalactose furnished polymeric substances.

Reversion products, known to be formed during the acidic treatment of carbohydrates, e.g. starch, may bear little structural similarity to the parent substance, and the identification of the linkage in a disaccharide obtained by chemical hydrolysis does not indicate, without further evidence, the occurrence of that linkage in the original polysaccharide.

The formation of reversion products can be represented diagrammatically as follows:



Reaction I represents the common hydrolytic cleavage of polyglycosides, favoured by low concentrations of carbohydrate, low concentration of acid and low temperatures. Sadovyi (1939) showed that the rate of hydrolysis was proportional to the concentration of acid present and the evaluation of the rate constants agreed well with experimental findings.

The condensation reaction II*A*, was studied by Moelwyn-Hughes (1929) using glucose and normal hydrochloric acid between 60 and 70°. In all cases an equilibrium between monosaccharide and condensation products was attained, the composition of which appears to be dependent on the initial concentration of glucose. Similar investigations were carried out by Seaman (1944), and it was pointed out by Silin & Sapegina (1939) that the elimination of water in reaction II*A* was an important step, permitting the application of a modified bimolecular equation to the system. The monosaccharides could be recovered by dilute acidic hydrolysis of the 'reversion products' (reaction II*B*). Under more drastic conditions irreversible intramolecular breakdown of the monosaccharide occurs (reaction III).

The researches of Berlin (1926) and Georg (1930) on reversion products of glucose indicate the presence of 1:6-linked disaccharides, particularly *iso*maltose (glucosido-α-1:6-glucose), whilst the occurrence of other disaccharides is discussed by Pringsheim (1924, 1926), Hurd & Cantor (1938) and others. The presence of non-reducing disaccharides and trisaccharides was shown by Hurd & Edwards (1949), and an extensive investigation of the condensation of partly substituted glucoses was made by Frahm (1941, 1944). It was found that whereas 1:2, 1:3 and 1:4 glucosidic linkages were formed at about the same rate, 1:6 linkages were formed with markedly greater readiness. By the action of 40% hydrochloric acid on glucose, Myrbäck, Hammarstand & Gelinder (1949) obtained a mixture of tetrasaccharides in which 1:6, 1:2 and/or 1:3 linkages were found.

Reversion products of a high degree of polymerization were described by Fredenhagen & Cadenbach (1933) who showed that cellulose reacted readily with liquid hydrogen fluoride in anhydrous conditions, forming glucosyl fluoride. In the presence of traces of water or removal of the hydrogen fluoride by evaporation, a fluorine-free polysaccharide material was obtained different from the initial cellulose. This new polymeric material termed 'cellan' (Helferich & Böttgers, 1929; Helferich, Stärker & Peters, 1930; Helferich & Peters, 1932) had weak reducing properties, was soluble in water and was strongly dextrorotatory. It was claimed that cellan was formed also by the action of hydrogen fluoride on glucose (Fredenhagen & Helferich, 1929). More recently Pacsu & Mora (1950) reported the formation of polymers of high molecular weight, non-dialysable in cellophan membranes by concentration of glucose with hydrochloric acid.

We have now synthesized polymers from a number of hexoses and mixtures of hexoses in order to study their physical and biochemical properties, in view of the interesting possibilities which the materials present.

EXPERIMENTAL

Conditions of polymerization. The monosaccharide was mixed with $SOCl_2$ by being ground in a mortar and the reactants, packed in a glass vessel, were heated at various temperatures in a thermostatically controlled oven (±2°). Whilst still hot the resulting pale-yellow liquid was dried in a vacuum and the crude product was obtained as a brittle sponge-like mass, which was water-soluble.

Reducing values (R.V.). The reducing values were determined by the method of Somogyi (1952) and expressed as the ratio of the weight of polymer to the weight of anhydrous glucose of the same reducing power.

Optical rotations. Measurements were made on a Hilger Standard microptic polarimeter in sodium light, readings being accurate to ±0·01°.

Chromatography. Examinations were carried out on Whatman no. 1 paper using butanol:ethanol:water (4:1:5, v/v) as a descending eluting agent as described by Partridge (1946). The behaviour of components is described in terms of R_G values (Hough, Jones & Wadman, 1949).

Fractionation by ethanolic precipitation. The material was dissolved in 50 ml. of water and ethanol was added dropwise, with stirring, to the solution at room temperature. The precipitate was removed centrifugally (3000 rev./min.) and was dried over absolute ethanol and ether. The addition of ethanol to the supernatant was resumed until a second precipitate was observed. This, and subsequent fractions obtained in the same way, was separated and dried.

Preparation of non-dialysable glucose polymers. (*a*) 18 g. glucose monohydrate were ground with 3 ml. of $SOCl_2$, heated at 105° for 45 min. After drying, the fraction precipitated by 3 vol. of ethanol was dried and heated at 50° for 1 hr. with 2 ml. HCl. The syrup so formed was dissolved in 100 ml. of water and dialysed in cellophan against distilled water for 36 hr. and again precipitated by 3 vol. of ethanol, yielding 2·1 g. of product (R.V., 52·8, $[\alpha]_D^{21} + 100°$ in water, *c*, 0·6).

(*b*) 3 g. of glucose polymers (R.V. 10) were mixed with 0·5 ml. $SOCl_2$ and heated at 105° for 45 min. The product dried *in vacuo* to a pale-yellow dry friable foam which, dissolved in 13 ml. of water, was dialysed in cellophan for 48 hr. and isolated by freeze drying. Yield, 0·4 g. (R.V. 33·2, $[\alpha]_D^{21} + 100°$ in water, *c*, 0·6.) Material (designated polyglucose A) prepared in this manner was used for further examination.

Hydrolysis with dilute mineral acid. 80 mg. of polyglucose A were heated at 100° with 10 ml. of 0·01 N-HCl. No change in reducing power or in optical rotation was observed after 3 hr. nor could monosaccharides be found by chromatographic analysis. The concentration of the acid was increased to 1N and the rotation changed $[\alpha]_D^{19} + 101° \rightarrow +72°$ (constant value) in 90 min.

Paper chromatography of the hydrolysate revealed a single reducing substance with properties corresponding to glucose, run simultaneously. Glucose was identified in the hydrolysate by the formation of glucosazone.

Sedimentation measurements. These were made over a range of concentrations using polyglucose A, in 0·1 M-NaCl, and the results are reported separately by Baldwin (1953).

Diffusion coefficient. This was determined by Mr J. R. Hall with polyglucose A using the Gouy diffusometer as described by Ogston (1949).

Viscosity. This was determined at 25° (±0·1°) in an Ostwald viscosimeter using a range of concentration of the fractions.

Action of enzymes. (*a*) *Amylase.* Freshly collected saliva was diluted with five volumes of 0·85% saline and centrifuged for 30 min. at 2000 rev./min. Samples of polyglucose A were incubated at 37° with 1 ml. of the enzyme preparation and the amount of reducing sugar generated was determined at intervals using the Somogyi method (1952). Control digests in which the polyglucose was substituted by corresponding amounts of whole potato starch were carried out simultaneously. (*b*) *Invertase.* Fresh baker's yeast (5 g.) was autolysed in the presence of toluene (10 ml.) for 8 days. The resulting mixture was centrifuged and the aqueous layer containing invertase was dialysed in cellophan and centrifuged before use. (*c*) *Potato phosphorylase.* Fresh potato juice at pH 6 was centrifuged for 20 min. at 0° before use.

RESULTS

In order to arrive at suitable conditions for the polymerization of hexoses, three series of experiments were performed.

(*a*) Glucose monohydrate was heated with varying amounts of $SOCl_2$ at 105° for 30 min. The results are shown in Table 1.

Table 1. *Action of thionyl chloride on* D-*glucose monohydrate* (18 *g.*)

(105° for 30 min.)

Preparation	Vol. $SOCl_2$ (ml.)	Yield (g.)	Reducing value
1	1·0	10·2	4
2	2·0	10·7	4·3
3	3·0	10·5	5·9
4	4·0	10·2	5·0
5	4·6	9·8	6·7
6	5·0	10·2	6·0

(*b*) Glucose monohydrate (18 g.) was heated at 105° with $SOCl_2$ (3 ml.) for varying times. The resulting fractions (Table 2) were dissolved severally in 25 ml. of water and the material, precipitated by the addition of 75 ml. of ethanol, was collected and dried.

Table 2. *Polymerization of glucose at varying times*

(105°, 18 g. glucose, 3 ml. $SOCl_2$.)

Preparation	Time (min.)	Yield of material precipitated with 3 vol. ethanol (g.)	Reducing value
7	30	13·7	10·8
8	45	12	9·9
9	60	11	11·8
10	90	10·7	7·8

(*c*) The effect of temperature on the polymerization was investigated, each experiment being carried out on 18 g. glucose monohydrate mixed with 3 ml. of $SOCl_2$ (Table 3).

Table 3. *Polymerization of glucose at various temperatures*

(18 g. glucose, 3 ml. $SOCl_2$.)

Preparation	Temp. (°)	Yield of material precipitated with 3 vol. ethanol (g.)	Reducing value
11	37	7·5	2·1
12	60	12·7	2·36
13	100	11	4·82
14	105	12	6·78
15	130	9·1	7·1
16	158	4·8	8·2

Paper-chromatographic examination of the crude polymeric material (preparation 3, Table 1) showed the presence of unchanged glucose and some disaccharide-like products, though the main bulk of material was immobile.

Fractionation by ethanolic precipitation showed that polymers constituting about 30 % of the whole, with a mean chain length of ten units, were precipitated by 3 vol. of ethanol (Fig. 1*a*), and that products of higher degrees of polymerization (D.P.) could be separated from this fraction by precipitation by graded amounts of ethanol (Fig. 1*b*). A considerable increase in the degree of polymerization was found and products which did not dialyse through cellophan resulted, when small-chain polymers (preparation 3, Table 1), fractionated with 3 vol. of ethanol and dried, were retreated with thionyl chloride under the same conditions as the initial polymerization. Yields of the dialysed product (polyglucose A) varied between 7 and 20 % of the small-chain polymers used. The product was a pale-yellow amorphous solid with a D.P. of 33·2. In an examination of the physical properties of polyglucose A, the diffusion constant (D_W^{20}) was found to be $12{\cdot}6 \times 10^{-7}$ and the sedimentation constant (S_W^{20}), $0{\cdot}80 \times 10^{-13}$. Assuming $\bar{V} = 0{\cdot}62$ these values combine to give a value of 4100 (±5 %) for the molecular weight. This value is in keeping with the magnitude of the intrinsic viscosity (0·018) of the material. The polymer was chlorine- and sulphur-free and when it was hydrolysed by 1N-HCl, only glucose could be identified (chromatographically,

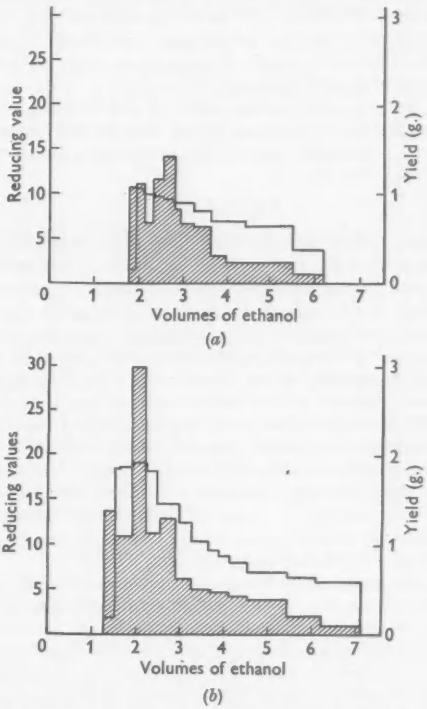


Fig. 1. (*a*) Fractionation of crude polysaccharide (18 g.). (*b*) Fractionation of polysaccharide (18 g.) with B.V.10. Hatched portions refer to reducing values.

Table 4. *Properties of synthetic hexose polymers*

Preparation	Weight of sugars (g.)	Vol. $SOCl_2$ (ml.)	Temp. (°)	Time (min.)	Fractionations (vol. ethanol)	Yield (g.)	Reducing value	$[\alpha]_D$
17	Galactose, 9 g. (+1 ml. water)	1·5	105	30	2	4·6	9·3	+70
					3	1·6	6·2	+93·5
18	D-Glucose monohydrate, 3 g., L-rhamnose, 3 g.	1·0	105	30	7	0·3	10·5	+171
19	Lactose, 9 g. (+0·5 ml. water)	1·5	105	30	2	5·1	12·1	+86
					3	0·7	7·9	+72
20	D-Mannose, 3 g. (+0·1 ml. water)	0·5	105	60	2	1·0	12·4	+64
					3	0·3	10·7	+60
21	D-Glucose monohydrate, 9 g. D-Galactose, 9 g.	3·0	105	30	2	10·3	10·8	+76
					3	1·8	—	+107
22	D-Glucose monohydrate, 1·0 g. D-Glucurone, 0·5	0·2	105	30	7	0·16	4·5	+79

as the osazone and as the benziminazole derivative). The polymer was not degraded by salivary amylase under conditions which hydrolysed a similar amount of starch in 5 min. The enzyme was not inactivated by the polyglucose. Similarly, yeast invertase and potato phosphorylase were without detectable action on polyglucose A.

By the same process used for the formation of polyglucose A, a number of simple and mixed hexose polymers were synthesized and characterized (Table 4).

DISCUSSION

The results indicate that the polymerization is facilitated by the presence of small amounts of water, and retarded by traces of alcohols or glycosides. When anhydrous hexoses were used, there was no evidence of polymerization to any marked extent. At temperatures below 100°, the rate of polymerization was slow and most effective results were obtained at the higher temperatures. Above 150° decomposition of the sugar occurred readily. The higher degrees of polymerization resulted from a re-treatment of small-chain polymers. Attempts to keep the sugar in contact with the catalyst for longer periods by means of hydrocarbon solvents (toluene, xylene) gave results little different from the repolymerization experiments.

Ethanol-water fractionation proved useful in separating the more highly polymerized material (D.P. 10 and greater) from unchanged glucose, disaccharides and other products. The degrees of polymerization have been calculated by measurement of residual reducing power in isolated fractions, in terms of glucose, and this value represents the D.P. only if the molecule has a single reducing end. These values have been taken as an index of the extent of polymerization. In the ultracentrifuge a broad peak was observed and certain consistency of properties was observed in the fractionation procedure, but no claim is made that the polyglucose A is a single polymeric species. Myrbäck *et al.* (1949) observed that a synthetic tetrasaccharide preparation was a mixture of at least three substances.

The identification of glucose as the sole product of acidic hydrolysis substantiates the view that in the polymer this sugar is glycosidically linked and that internal elimination of the elements of water forming anhydro-sugar units (Newth, Overend & Wiggins, 1947; Peat, 1946) does not occur. The stability of the polymer towards dilute acids is more characteristic of pyranoside than furanoside or acyclic acetal linkages, and the change in optical rotation ($[\alpha]_D^{19} + 101° \rightarrow +72°$) during hydrolysis suggests the occurrence of some α-linkages. The polymer was remarkably stable towards enzymic hydrolysis by salivary amylase, invertase and potato phosphorylase. The enzymes were not inhibited by the polymeric material.

Insufficient evidence is available to shed much light on the mechanism of polymerization but the dehydrating conditions required suggest that the concentration of water plays a significant part. The acidic catalyst may lead to the formation of glycosyl complex as in the case described by Pacsu & Hillier (1949). The number of simple and mixed polymers synthesized from hexoses other than glucose indicates that the reaction is of a general nature.

SUMMARY

1. Conditions of temperature, time and concentrations are described under which glucose, in the presence of thionyl chloride, forms polymeric products.

2. Non-dialysable glucose polymeric material (molecular weight, 4100) was found to have D_w^{20} $12{\cdot}6 \times 10^{-7}$, S_w^{20} $0{\cdot}80 \times 10^{-13}$, and $[\eta]$ 0·018.

3. The polymer ($[\alpha]_D^{21} + 100°$ in water) was hydrolysed completely by 1N-hydrochloric acid to glucose, but was not degraded by salivary amylase, invertase or potato phosphorylase.

I wish to thank Mrs J. Maskins for her invaluable technical assistance in these investigations and Prof. Sir Rudolph Peters, F.R.S., for his interest and encouragement.

REFERENCES

Baldwin, R. L. (1953). *Biochem. J.* (in the Press).
Berlin, H. (1926). *J. Amer. chem. Soc.* **48**, 1107.
Deriaz, R. E. (1949). *J. chem. Soc.* p. 2856.
Drew, H. D. K. & Haworth, W. N. (1927). *J. chem. Soc.* p. 775.
Fischer, E. (1890). *Ber. dtsch. chem. Ges.* **23**, 3687.
Fischer, E. (1898). *Ber. dtsch. chem. Ges.* **28**, 3024.
Frahm, H. (1941). *Ber. dtsch. chem. Ges.* **74**B, 622.
Frahm, H. (1944). *Liebigs Ann.* **555**, 187.
Fredenhagen, K. & Cadenbach, G. (1933). *Angew. Chem.* **46**, 113.
Fredenhagen, K. & Helferich, B. (1929). *D.R.P.* **521**, 340. Quoted in *Chem. Abstr.* **23**, 1523.
Georg, A. (1930). *C.R. Soc. Phys. Hist. nat. Genève*, **47**, 94.
Helferich, B. & Böttgers, S. (1929). *Liebigs Ann.* **476**, 150.
Helferich, B. & Peters, O. (1932). *Liebigs Ann.* **494**, 101.
Helferich, B., Stärker, A. & Peters, O. (1930). *Liebigs Ann.* **482**, 183.
Hough, L., Jones, J. K. N. & Wadman, W. H (1949). *J. chem. Soc.* p. 2511.
Hurd, C. D. & Cantor, S. M. (1938). *J. Amer. chem. Soc.* **60**, 2677.
Hurd, C. D. & Edwards, O. E. (1949). *J. org. Chem.* **14**, 680.
Moelwyn-Hughes, E. (1929). *Trans. Faraday Soc.* **25**, 86.
Myrbäck, K., Hammarstand, M. & Gelinder, H. (1949). *Ark. Kemi*, **1**, 235.

Newth, F. H., Overend, W. G. & Wiggins, L. F. (1947). *J. chem. Soc.* p. 10.
Ogston, A. G. (1949). *Proc. Roy. Soc.* A, **196**, 272.
Overend, W. G., Shafizadeh, F. & Stacey, M. (1951). *J. chem. Soc.* p. 994.
Pacsu, E. & Hillier, J. (1949). *J. Amer. chem. Soc.* **70**, 523.
Pacsu, E. & Mora, P. T. (1950). *J. Amer. chem. Soc.* **72**, 1045.
Partridge, S. M. (1946). *Nature, Lond.*, **158**, 270.
Peat, S. (1946). *Advanc. carbohyd. Chem.* **2**, p. 37. N.Y.: Academic Press.
Pringsheim, H. (1924). *Ber. dtsch. chem. Ges.* **57**, 884, 1581.
Pringsheim, H. (1926). *Ber. dtsch. chem Ges.* **59**, 1001.
Sadovyĭ, A. (1939). *Trudy Voronezh. Khim.-Tekhnol. Inst.* **3–4**, 89.
Seaman, J. F. (1944). *Report No.* R. 1457. Forest Products Lab., U.S. Dept. Agriculture, Madison.
Silin, P. M. & Sapegina, E. A. (1939). *Trudy Voronezh. Khim.-Tekhnol. Inst.* **3–4**, 79.
Somogyi, M. (1952). *J. biol. Chem.* **195**, 19.

The Component Acids of Milk Fats of *Balaenoptera physalus* (Finner Whale)

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Although the component acids of the milk fats of many land animals, especially ruminants, are characterized by the presence of acids of lower molecular weight than those present in their respective depot fats, the component acids of milk fats of marine mammalia so far examined are qualitatively and to a greater or lesser extent quantitatively similar to those of their blubber fats. Thus Schmidt-Nielsen & Frog (1933) were unable to identify any acids of lower molecular weight than lauric in the component acids of milk fat of finner whale (*Balaenoptera physalus*) and showed that the proportions of the chief groups of acids (saturated and unsaturated together) were: C_{14}, 5·5; C_{16}, 22; C_{18}, 32; C_{20-22}, 39. These results, taken in conjunction with the iodine value of the oil (137·8) and the presence of palmitoleic, oleic, gadoleic, and 'clupanodonic' acids, were considered to indicate the close similarity between the milk and blubber fats. Similar figures reported by Klem (1935) for the milk fat of the blue whale (*Balaenoptera musculus*) are in agreement with this view. The values obtained by Klem are nevertheless somewhat speculative, since he reported the loss of a fraction comprising about 24% of the mixed acids, and was obliged to make tentative assumptions as to the composition of this fraction. In spite of this indeterminacy the results obtained by Klem are in good agreement with those obtained in the present work. Meara (1952), however, showed that although the component acids of seal milk fat were broadly similar to those of seal blubber fat there were certain significant differences notably in the palmitic acid contents.

The present investigation, which was made possible by Dr J. C. D. White, who kindly placed four specimens of finner-whale milk at our disposal, was carried out to determine how far the broad similarity between the milk and blubber fats holds, and to determine whether any significant differences similar to those which obtain in the case of seal milk and blubber fatty acids also occur in whale milk and blubber fats respectively.

METHODS AND RESULTS

The following data supplied by Dr White indicate the origin of the milk from which the fats were extracted. For each finner whale the length, and the time after death at which the sample was taken, are given. *A*: 72 ft. (23·5 m.), 7 hr. 45 min.; *B*: 68 ft. (22·1 m.), 6 hr.; *C*: 69 ft. (22·3 m.), 16 hr. (sample obtained from nipple and probably purest sample); *D*: 73 ft. (23·7 m.), 19 hr. (whale appeared to be drying off; a little blood present from external source).

The samples were viscous, resembling condensed milk in consistency, pale pink in colour, and appeared to be in good condition, with the exception of specimen *D*, which had a highly rancid fishy odour and was contaminated with blood and aqueous medium. Each sample was extracted in the manner described by Meara (1952) for seal milk fat, the extraction data together with the chemical characteristics of the extract being given in Table 1.

The component acids of each milk fat were determined as reported by Meara (1952). Unsaponifiable matter was removed from the soaps after saponification of the fat. Steam distillation of the regenerated fatty acids, followed by titration of the steam distillate, indicated the absence of the lower molecular weight steam-volatile fatty acids. The recovered acids were resolved by low-temperature crystallization into groups which consisted of mainly saturated, mainly monoethenoid, and mainly polyethenoid acids, respectively, each group being methylated (Bjarnason & Meara, 1944) and fractionally distilled. From the analytical data obtained the composition of each ester fraction was computed (Hilditch, 1947, pp. 498–510); thence the composition of each group of acids, and finally the composition of the whole fat was deduced.

The final results of these analyses, together with data on related fats, are given in Table 2.

Table 1. *Extraction and analytical data of whale milks and fats*

Extraction of milks

Specimen	*A*	*B*	*C*	*D*
Milk (g.)	211	348	349	115
Fat extracted (g.)	115·0	140·5	129·4	26·38
(%)	54·5	40·4	37·1	22·9
Solid residue (g.)	21·0	17·0	39·0	23·0
(%)	9·9	4·9	11·2	20·0
Water content (g.)	75·2	190·5	180·6	65·6
(%)	35·6	54·7	51·8	57·0

Analytical data for milk fats

Specimen	*A*	*B*	*C*	*D*
Saponification equivalent	295·5	295·4	297·3	293·5
Iodine value	170·3	177·3	149·0	162·1
Free fatty acids (% as oleic acid)	0·7	0·7	0·6	1·1
Unsaponifiable matter (%)	0·9	0·7	1·2	1·8
Iodine value of unsaponifiable matter	168·0	169·0	124·5	169·6

Table 2. *Component fatty acids of whale and seal milk and blubber fats*

(All values are %, w/w.)

	Finner-whale milk (Present investigation)				Blue-whale milk (Klem, 1935)	Whale blubber (Hilditch & Maddison, 1948)	Grey seal	
	A	*B*	*C*	*D*			Milk (Meara, 1952)	Blubber (Cardin, 1952)
Iodine value of fat	170·3	177·3	149·0	162·1	171·8	95·0	146·2	157·9
Acid								
Lauric	0·7	—	—	—	—	Trace	—	—
Myristic	7·1	7·3	10·0	2·4	8·4	9·2	2·8	3·4
Palmitic	15·8	16·6	17·6	14·0	16·8	15·6	16·4	8·9
Stearic	3·7	2·9	3·4	3·1	1·8	1·9	2·8	1·8
Arachidic	—	0·8	0·3	—	—	0·6	—	0·2
Unsatd. C_{14}	1·0	2·4	1·4	0·5	1·2	2·5	1·7	2·0
Unsatd. C_{16}	7·9	6·2	8·1	6·7	6·2	13·9	12·7	15·6
Unsatd. C_{18}	29·7	29·7	26·6	37·2	26·8	37·2	36·6	33·3
Unsatd. C_{20}	18·2	19·1	17·6	21·5	25·9	12·0	13·6	18·7
Unsatd. C_{22}	15·9	15·0	15·0	14·6	12·9	7·1	13·4	16·0
Mean unsaturation of								
Unsatd. C_{14}	−2·0	−2·0	−2·0	−2·0	−2·0	−2·0	−2·0	−2·0
Unsatd. C_{16}	−2·2	−2·7	−2·8	−2·9	−2·0	−2·1	−2·2	−2·2
Unsatd. C_{18}	−2·8	−3·3	−2·4	−2·8	−3·3	−2·4	−2·3	−2·5
Unsatd. C_{20}	−7·9	−8·2	−7·6	−6·0	−8·5	−7·1	−6·6	−6·2
Unsatd. C_{22}	−11·0	−10·8	−10·1	−10·9	−11·0	−9·4	−11·2	−10·1

DISCUSSION

The pink coloration of whale milk appears to be due to natural pigmentation and not to contamination from the mammary glands, from which the material of specimens *A* and *B* were obtained, since the specimen *C* drawn from the nipple was equally pink in colour.

Clowes (1929) indicated that considerable difficulties arise in the sampling of whale milk. If the milk is obtained by dissection of the mammary glands, blubber oil and blood are possible contaminants. Specimen *D* was contaminated in this way, whereas specimens *A* and *B*, also obtained by dissection of the mammary gland, showed no signs of contamination.

In order to obtain a representative sample of the milk of ruminants it is desirable to take the whole content of the milk reservoir. Thus the 'fore-milk' drawn from the lower part of a cow's udder may contain only 1·7 % of fat while the last drawn 'strippings' may contain 4–10 % of fat. It is not known, however, whether the fats from 'fore-milk' and 'strippings' differ in composition to any marked extent; though Baldwin & Longenecker (1944) have shown that cow colostrum fat differs slightly from milk fat produced later in the lactation period. In the present instance where the milks have a high viscosity it is unlikely that creaming of the milk, or preferential segregation of the glycerides, occurs.

In this discussion only specimens *A*, *B* and *C* will be considered, since specimen *D* being rancid and

contaminated cannot be perhaps regarded as representative. A further limitation is that small samples of highly complex fats cannot be analysed as accurately as larger specimens. The differences between the results obtained for specimen *D* and the other two fats of approximately the same iodine value (specimens *A* and *B*) are, however, considerably in excess of any expected experimental error.

Table 2 includes the component fatty acids of the milk fat of blue whales reported by Klem and the component fatty acids of a commercial specimen of whale oil (Hilditch & Maddison, 1948). Whilst the latter specimen consists predominantly of that of the blue whale, these values may be regarded as typical of commercial Antarctic-whale blubber oils, since it is known (Armstrong & Allan, 1924; Tveraaen, 1935; Hilditch & Maddison, 1948) that the composition of whale oils varies but little from season to season. The possibility of slight variation in composition of the blubber fat with species differences, however, cannot be completely eliminated. The table also includes data for the composition of milk and blubber fats of the grey Atlantic seal (*Halichoerus grypus*).

It is apparent from the table that no such marked divergency occurs between the component fatty acids of the milk and blubber fats of the whale as occurs between the milk and depots fats respectively of the ruminants and other land animals. The saturated-acid contents of the present milk fats are in remarkably close agreement with those obtained by Klem (1935) for the milk fat of the blue whale, and with those recorded by Hilditch & Maddison (1948) for whale blubber fat. These values are in marked contrast to those in seal milk and blubber fats; here the palmitic acid content of the milk fat (16·4 %) is about twice that (8·9 %) of the blubber fat. There is, however, little difference between the unsaturated acids of seal milk and blubber fats.

Although the whale milk and blubber fats are practically identical in their saturated acid contents, this is not so for the unsaturated acids. Thus, while the unsaturated C_{14} acid content remains approximately constant in all the fats, the milk fats contain about 6–8 % C_{16} unsaturated acids whilst the blubber fat contains 14 %. Similarly, the percentage content of C_{18} unsaturated acids in the milk fat is about 6–7 units lower than that in the blubber. On the other hand, milk fat contains more C_{20} and C_{22} unsaturated acids than blubber fat.

The low iodine value of the fat from specimen *C* is not apparently due to any one particular cause. The total content of saturated acids is only 4–5 % higher than that of specimens *A* and *B*; this is due to an increase in the myristic and palmitic acid content at the expense primarily of the unsaturated C_{18} and C_{20} acids, the mean unsaturations of which are also significantly lower.

Though broadly speaking the milk fats of the marine mammalia are similar to their respective blubber fats, it may be difficult to arrive at a simple correlation between them. It is probable, however, that a simpler relationship exists between the milk and blubber fats of marine mammalia than obtains in the milk and depot fats of land animals. The iodine values in Table 2 show that the mean unsaturation of whale milk fat is greater than that of the blubber fat, while the converse is true in the case of the seal. If in marine mammalia the milk fat is derived more or less directly from the blubber fats, it follows that a preferential mobilization of the more unsaturated components of the blubber fat has taken place. This would result in the blubber of a lactating whale being somewhat richer in the more saturated components, the blubber fat therefore having a lower iodine value. Klem (1935) has reported lower iodine values for the blubber fats of lactating whales than for the fats from the same sites in healthy males and non-lactating females. The lactating seal, however, appears to metabolize the more saturated components of the blubber for milk production thereby increasing the mean unsaturation of its blubber fat. Unfortunately no adequate data are available for the comparison of the blubber fats of the lactating and non-lactating seal since it is now well established (Hilditch & Pathak, 1949; Winter & Nunn, 1950*a*, *b*, *c*; Cardin, 1952) that seal blubber fats vary considerably in composition depending among other things on diet, sex and age.

SUMMARY

1. Finner-whale (*Balaenoptera physalus*) milks resemble that of the grey Atlantic seal (*Halichoerus grypus*) in that the fat content is of the order of 10 times that of the milk of land animals.

2. Whale milk fat contains only those acids which occur in the blubber fat; though there is a broad similarity between milk and blubber fats, differences occur in the proportions of the unsaturated acids.

3. Whale milk fat is more unsaturated than the blubber fat; the reverse is true for lactating seals. It is suggested that this is due to selective mobilization of blubber fat for milk production.

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REFERENCES

Armstrong, E. F. & Allan, J. (1924). *J. Soc. chem. Ind., Lond.*, **43**, 216T.

Baldwin, A. R. & Longenecker, H. E. (1944). *J. biol. Chem.* **155**, 507.

Bjarnason, O. B. & Meara, M. L. (1944). *J. Soc. chem. Ind., Lond.*, **63**, 61.

Cardin, A. (1952). Ph.D. Thesis, University of Liverpool.

Clowes, A. J. (1929). *Discovery Reports*, vol. 1, p. 472. Cambridge University Press.

Hilditch, T. P. (1947). *The Chemical Constitution of Natural Fats*, 2nd ed. London: Chapman and Hall.

Hilditch, T. P. & Maddison, L. (1948). *J. Soc. chem. Ind., Lond.*, **67**, 253.

Hilditch, T. P. & Pathak, S. P. (1949). *Biochem. J.* **44**, 218.

Klem, A. (1935). *Hvålrad. Skr.* **11**, 49.

Meara, M. L. (1952). *Biochem. J.* **51**, 190.

Schmidt-Nielsen, S. & Frog, F. (1933). *K. norske vidensk. Selsk. Forh.* **6**, 127.

Tveraaen, I. (1935). *Hvålrad. Skr.* **11**, 5.

Winter, G. & Nunn, W. J. (1950*a*). *J. Sci. Fd Agric.* **1**, 18.

Winter, G. & Nunn, W. J. (1950*b*). *J. Sci. Fd Agric.* **1**, 311.

Winter, G. & Nunn, W. J. (1950*c*). *J. Sci. Fd Agric.* **1**, 314.